Von Willebrand Factor:

clearance as regulator of plasma levels

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Von Willebrand Factor: klaring als regulator van plasma concentratie
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Contents

CHAPTER 1

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General Introduction

Von Willebrand Factor during haemostasis and coagulation

Haemostasis is the process that maintains the integrity of the mammalian circulatory system in response to vascular damage and refers to the complex interaction between vessels, blood cells, coagulation factors, coagulation inhibitors and fibrinolytic proteins. The goal of the haemostatic system is to maintain blood in a fluid state within the vascular compartment. After damage of a blood vessel the equilibrium is disturbed and several steps occur to restore the balance between hemorrhage and thrombosis. This response must be quick, localized and carefully regulated. First, vasoconstriction narrows the blood vessel thereby minimizing the vessel diameter and slowing bleeding. Rupture of the vessel wall causes exposure of collagen from the underlying tissue. Subsequently, platelets interact with vessel wall components to start formation of a haemostatic plug [1]. Following platelet binding, the coagulation cascade is initiated. During coagulation, a small stimulus at the beginning of the enzymatic cascade is catalytically amplified at each step by coagulation factors. The final effect is the transformation of fibrinogen into polymerized fibrin, which stabilizes the platelet plug [2]. When the vessel wall is repaired, fibrinolysis facilitates dissolvement of fibrin. Fibrinolysis is the process that converts plasminogen into plasmin, wich is the enzyme that proteolytically degrades fibrin [3].

One of the plasma proteins that plays a role in haemostasis and coagulation is von Willebrand Factor (VWF). In plasma, VWF circulates as a multimeric protein in an inactive state. However, binding to exposed subendothelial matrix, together with high shear stress converts VWF into its platelet-binding conformation. Now VWF can serve as a molecular bridge between the exposed matrix and platelets. This interaction is of importance, since platelets are unable to bind this matrix under high shear stress in the absence of VWF [4].

The contribution of VWF to coagulation also concerns the stabilisation of factor VIII (FVIII) [5-7]. FVIII is an essential element of the coagulation cascade, where it acts as a cofactor for activated factor IX (FIX) [8]. Complex formation between FVIII and VWF stabilizes the heterodimeric structure of FVIII and premature clearance from the circulation is prevented [8,9].

VWF is also implicated in several other processes, like smooth muscle cell proliferation, metastasis and recruitment of leukocytes [10-12]. Moreover, the interaction between *Staphylococcus aureus* surface proteins and VWF contributes to the adherence of the bacterium to platelets or to damaged blood vessels [13,14].

Synthesis and structure of VWF

VWF is produced in endothelial cells and megakaryocytes as a single pre-pro-polypeptide [15,16]. Monomeric VWF is composed of four repeated domains (A-D) that are arranged in the sequence: D'-D3-A1-A2-A3-D4-B1-B2-B3-C1-C2-CK (Figure 1A) [4]. Formation of large VWF multimers is a complex proces (Figure 1B).

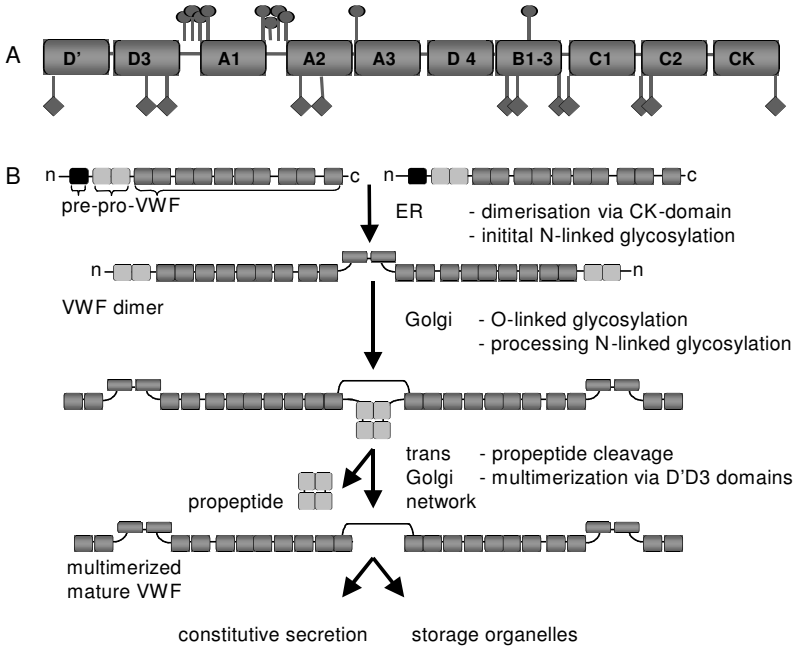

Figure 1: Structure and synthesis of VWF.
A: Structure of mature VWF. Indicated are the four repeated domains (A-D) and sites of glycosylation. Circles above the domains represent the O-linked glycosylation sites, while squares below the domains show the location of N-linked glycosylation sites. **B:** Biosynthesis of VWF. In the endoplasmatic reticulum (ER) the VWF monomers are dimerised via disulphide bonds between the CK domains and N-linked glycosylation is initiated. Once in the Golgi, the N-linked glycans are further processed and O-linked glycosylation is started. In the trans Golgi, dimers form multimers through disulphide bonds between their D'D3 regions, which is facilitated by the cleaved propeptide (D1-D2). Finally, fully functional VWF multimers are either secreted in plasma or stored in the cell.

After removal of the signal peptide, proVWF is translocated to the endoplasmatic reticulum (ER) were it undergoes initial N-linked glycosylation. In addition, proVWF-dimers are formed by disulphide bonds between the C-terminal CK domains of the monomers (Figure 1B) [4,17-19]. proVWF dimers are then transported to the Golgi apparatus where N-linked glycosylation is completed and O-linked glycosylation is applied [20]. Each VWF monomer contains 12 N-linked and 10 O-linked carbohydrate chains, that add 19% to the weight of VWF [21-23] (Figure 1A). The N-linked carbohydrates are composed of a variety of saccharide groups, while the O-linked carbohydrates predominantly consists of the tetrasaccharide structure called the sialylated T-antigen, which is a synonym for Galactose-β1,3-N-acetyl-galactosamine containing two sialic acid residues (NeuAc(α2-3)Gal(β1-3)(NeuAc(α2-6))GalNAc) [22,24] (Figure 2).

In the trans Golgi network, the propeptide (domains D1 and D2) is proteolytically removed and multimerization via binding of VWF-D'D3 domains is facilitated by the cleaved propeptide [25,26]. The multimeric structure of VWF is maintained by disulphide bonds between cysteines in the separate monomers (Figure 1B).

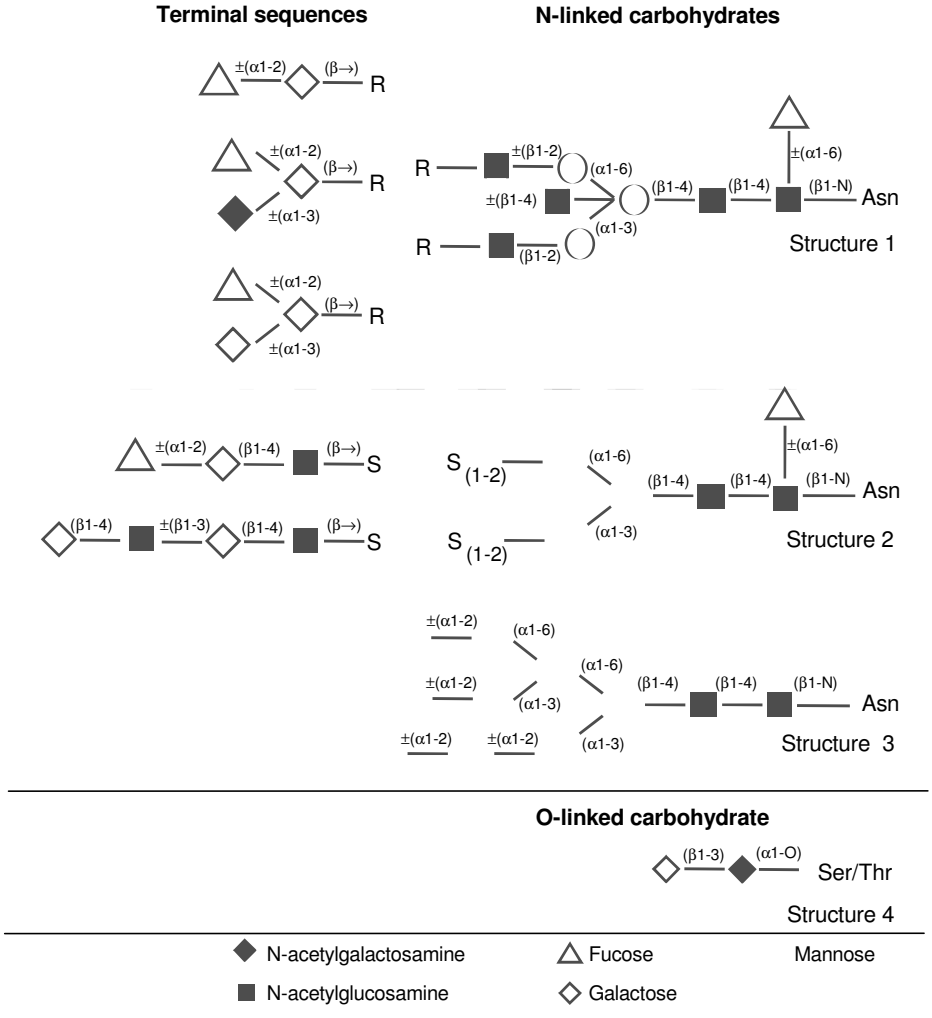

Figure 2: Desialylated carbohydrate structures on VWF.
VWF contains 12 N-linked glycosylation sites. Structure 1 may contain group R, whereas structure 3 may contain group S. The 10 O-linked glycosylation sites are composed of structure 4. Structure 1, 2 and 4 may be terminated with sialic acid residues at the terminal galactose, N-acetylgalactosamine and N-acetylglucosamine. (± indicates that some chains terminate at the site of the preceding sugar.).

VWF multimers are either secreted directly into plasma or stored in storage organelles: Weibel Palade bodies in endothelial cells or α-granules in megakaryocytes. VWF multimers and propeptide are released simultaneously in plasma in a 1:1 molar ratio [27,28]. Exocytosis of VWF from the storage organelles is tightly organized and may be initiated upon stimulation of the cells with an agonist [29]. After secretion of VWF in plasma, large multimers are cleaved into smaller derivatives by a metalloprotease called ADAMTS-13. Proteolysis of VWF by ADAMTS-13 is a well regulated process that results in VWF multimers of different sizes [30-32].

Von Willebrand Disease

Deficiency of VWF leads to von Willebrand Disease (VWD), a disease that was first described by Erik Adolf von Willebrand in 1926 [33]. VWD is a bleeding disorder and characterized by defects in blood clotting and formation of platelet plugs at sites of vascular injury. VWD is inherited in an autosomal dominant manner and can be divided into two general categories based on qualitative or quantitative defects within the VWF protein [34].

Total disruption of gene function by deletion of a segment of the gene or a nonsense mutation (abrupt termination of the protein by insertion of a stop codon into the coding sequence) results in quantitative VWF abnormalities [35-38]. Quantitative VWF deficiency is very diverse, with a mild to pronounced outcome of the disease. Patients with VWD type 3 suffer from a pronounced bleeding disorder with very little or no detectable plasma or platelet VWF and these patients have a secondary deficiency of FVIII [39,40]. While VWD type 3 is a relatively rare disease (approximately 1/1 million) [41], VWD type 1 is the most common form of VWD (70-80 % of the cases). VWD type 1 is the mild form of quantitative VWF deficiency, in which patients display decreased VWF levels (1-40% of normal) leading to highly variable phenotypes [42-44]. Low VWF plasma levels in these patients are the result from mutations, leading to decreased synthesis, impaired secretion, increased clearance or a combination of these conditions. Several studies showed that in more severe VWD type 1 cases, genetic changes are common within the VWF gene and highly penetrant. Genetic determinants in milder VWD type 1 cases involve factors in- and outside the VWF gene [45,46].

VWD type 2 is the outcome of a qualitative VWF deficiency in which a mutation in the VWF gene results in single amino acid substitutions that interfere with protein structure or function [44]. VWD type 2 is divided into 4 subclasses: type 2A, 2B, 2M and 2N. VWD type 2A is the most common qualitative abnormality of VWF and is often caused by missense mutations within the VWF-A2 domain. It is associated with selective loss of large and intermediate-sized VWF multimers. The reduction of larger multimers is caused by either increased sensitivity to proteolysis by ADAMTS-13 or impaired multimer assembly within the Golgi, leading to retention of the abnormal VWF in the ER [47,48]. VWD type 2B is also characterized by loss of larger multimers. However, in VWD type 2B this due to increased affinity of mutated VWF for the platelet GPIb-IX-V complex [49]. Gain-of-function mutations in the VWF-A1 domain lead to VWF with an abnormal conformation, which 'spontaneously' reacts with circulating platelets [50,51]. VWD type 2M includes variants in which platelet adhesion is impaired, due to abrogated binding to GPIbα (i.e. the opposite of the VWD type 2B mutation). However, the VWF multimer distribution in these patients is normal [52]. The mutations in the VWF gene responsible for VWD type 2N cause defective binding to FVIII [53,54]. FVIII from these patients is not stabilized by VWF and the clinical symptoms of these patients mimic Haemophilia A [55].

Haemophilia A

Haemophilia A is a severe bleeding tendency associated with functional absence of FVIII [8]. A well-known carrier of this X-linked recessive disease was Queen Victoria of England and through different marriages the mutant gene spread to royal families of Germany, Russia and Spain [56]. The level of residual FVIII cofactor activity in the blood determines the severity of the disease. FVIII levels of less than 1% are found in patients with severe haemophilia A. The major problem is painful bleeding within muscles or joints (mainly the knees, ankles and elbows). These bleeds may occur spontaneously and may result in permanent arthritis and disability if not treated promptly [57]. FVIII levels of 1-5% and spontaneous bleeds characterize moderate haemophilia A. Whereas, patients with mild haemophilia A display FVIII levels between 5-50% and they usually only experience bleeds following trauma or surgery.

The FVIII-VWF complex

FVIII is a glycoprotein of approximately 330 kDa with the domain structure A1-A2-A3-C1-C2. In plasma FVIII (about 95% of total) circulates in complex with VWF (about 5% of total). Upon proteolytic cleavage by thrombin, activated FVIII (FVIIIa) is released from VWF. FVIIIa acts as a cofactor for activated factor IX (FIX) in the coagulation cascade. FVIIIa is an unstable heterotrimer and subjected to either rapid inactivation through dissociation of the A2 subunit or inactivation by activated protein C [8].

The half-life of FVIII is 2 to 3 hours. However, in complex with VWF, the half-life of FVIII increases to 12 hours on average, which is similar to that of VWF [58,59]. Thus, maintenance of normal levels of FVIII in the circulation is dependent on complex formation with VWF. FVIII binds VWF with a high affinity (K_d=0.2 to 0.4 nmol/L) [60,61]. The binding site on VWF for FVIII resides within the amino terminal part of VWF, between residues 1 to 272 [23,62,63]. VWF binding to FVIII involves both the amino- and carboxyterminal region of the light chain [8,23,64-67].

Determinants of VWF plasma levels

The concentration of VWF in plasma is approximately 10 µg/ml. However, VWF plasma levels in the normal range vary widely and a number of factors that influence VWF plasma levels have been determined (Table 1). About 66% of total variation in plasma VWF levels is genetically determined and approximately 30% of this genetic component can be explained by ABO blood group. Average VWF plasma levels for persons carrying blood group O are 25% to 35% below that of persons with other blood groups [68-72]. Another hereditary determined modification of the glycosylation profile, called Secretor system, affects VWF plasma levels as well. Persons with blood group O and *SeSe*-genotype have significantly higher VWF levels when compared to those with blood group O in combination with *Sese*- or *sese*-genotype [73,74]. However, Schleef *et al* could not detect significant differences of VWF plasma levels between *SeSe*, *Sese* and *sese* individuals [75].

Table 1: Determinants of VWF plasma levels

Effector on VWF clearance	Observation	Proposed mechanism	Ref:
ABO blood group determinants	Individuals with blood group O display 25% decreased VWF levels	Blood group dependent clearance rate	58-61
Secretor system	*Se/Se* individuals show decreased VWF levels	Not known	62-64
Sialylation	Reduced sialylation results in decreased VWF levels	Increased VWF clearance	65;66
N-actetyl-galactosaminyl-transferase Galgt2/ B4galnt2	RIIIS/J mice demonstrate VWF enriched with N-acetylgalactosamine	Increased VWF clearance	67-69
O-linked carbohydrates	VWF devoid of O-linked carbohydrates was cleared more rapidly	Increased VWF clearance	70
Mutations or polymorphisms in the VWF gene	Mutations in VWF lead to differences in multiple VWF variables	Impaired VWF synthesis and/or clearance	40; 71-75

(Adapted from [76])

Several reports show that alterations in the carbohydrate moiety on VWF lead to decreased VWF plasma levels, due to increased clearance of affected VWF. Sodetz *et al* showed that enzymatic removal of sialyl-groups from carbohydrates on VWF, reduced circulatory half-life in rabbits 48 fold [76]. Furthermore, survival of endogenous VWF was reduced 2 fold in mice lacking ST3Gal-IV (an enzyme that connects sialylgroups to terminal galactose-residues on proteins) and reduced VWF plasma levels correlated with reduced sialylation in patients referred to the hospital for a suspected bleeding disorder [77]. Another link between carbohydrate moiety of VWF and its survival is displayed in RIIIS/J mice that show reduced VWF levels when compared to other strains. The gene responsible (*Galgt2/ B4galnt2*) was shown to encode α1,4N-acetylgalactosaminyltransferase. Murine expression of this glycosyltransferase is normally restricted to kidney and intestine. Aberrant expression in the endothelium of the RIIIS/J mice was shown to cause abnormal carbohydrate antigen expression on VWF, which resulted in a significantly increased rate of hepatic clearance [78-80]. As mentioned, VWF is also subjected to O-linked glycosylation. Currently, little is known concerning its effect on clearance of VWF. Recombinant VWF devoided of O-linked carbohydrates has a reduced half-life when administered to rats [81]. This may indicate that O-linked glycans have a protective effect on VWF survival. Alternatively, reduced levels of O-linked glycans may result in uncorrect folding of the protein. That would result in a reduced half-life, which is the consequence of suboptimal folded VWF.

Aim of this thesis

The mechanisms involved in mediating VWF clearance from plasma have not been well characterized. In humans VWF is cleared from the blood stream with a half life of 12-20 hrs [82-84]. Infusion studies with radiolabeled VWF in animal models have demonstrated that VWF is cleared principally by liver with a rapid initial phase followed by a slower secondary phase [76,81,85]. It is unclear however whether specific cells or receptors mediate hepatic uptake of VWF.

The objective of the studies described in this thesis was to investigate the process of VWF clearance. In order to gain more insight in parameters responsible for VWF plasma levels we designed the following research questions:

1 Does the extent of O-linked glycosylation on VWF determine VWF plasma levels? (Chapter 2)
2 What is the effect of cysteine mutations found in patients with VWD on the *in vivo* survival of VWF? (Chapter 3)
3 Does VWF half-life predict FVIII half life? (Chapter 4)
4 Which cell type contributes to the removal of VWF from the circulation? (Chapter 5)
5 What is the role of VWF in the adherence of bacterium to platelets? (Chapter 7)

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CHAPTER 2

CHAPTER 2

Variations in glycosylation of von Willebrand factor with O-linked sialylated T-antigen are associated with its plasma levels

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Abstract

The glycosylation-profile of von Willebrand factor (VWF) is known to strongly influence its plasma levels. VWF contains several carbohydrate structures, including O-linked glycans that primarily consist of sialylated T-antigen (NeuAc(α2-3)Gal(β1-3)[NeuAc(α2-6)]GalNAc). It is unknown yet if O-linked carbohydrates affect VWF levels. We developed an immunosorbent-assay based on neuraminidase-incubation allowing subsequent binding of Peanut-Agglutinin (PNA) to de-sialylated O-linked T-antigen on VWF. An inverse relation was found between PNA-binding and VWF-antigen levels in healthy individuals (n=111; Pearson-rank=-0.43; $p<0.0001$). A similar inverse association was observed in randomly-selected plasma-samples from our diagnostic laboratory: 252±125% for VWF<0.5 U/ml (n=15); 131±36% for VWF between 0.5 and 1.5 U/ml (n=32); 92±40% for VWF>1.5 U/ml (n=19). Reduced or increased PNA-binding was also observed in patients with increased (liver cirrhosis) or reduced (von Willebrand disease (VWD)-type 1) VWF-antigen levels, respectively. VWD-type 1 patients further displayed increased ratios of propeptide over mature VWF-antigen levels (0.38±0.18 versus 0.17±0.03 for patients and controls, respectively; $p<0.0001$), which is indicative for reduced VWF survival in these patients. Interestingly, a linear relation between PNA-binding and propeptide/VWF ratio was observed (Spearman-rank=0.47), suggesting a potential association between O-linked glycosylation and VWF survival. Finally, we detected a marked decrease in PNA-binding in post-DDAVP samples from various patients, indicating that the O-linked glycosylation profile of VWF stored in endothelial storage-organelles may differ from circulating VWF.

Introduction

Von Willebrand factor (VWF) is a multimeric glycoprotein that plays a dual role in the haemostatic process. First, VWF forms a complex with coagulation factor VIII, which enhances factor VIII survival in vivo. Second, VWF contributes to platelet adhesion and aggregation by acting as a molecular bridge between subendothelial collagen and platelets [1,2]. The relevance of VWF for the haemostatic system is apparent from the notion that deficiency or dysfunction of VWF is associated with von Willebrand disease (VWD), the commonest inherited bleeding disorder [3]. VWD can be categorized in qualitative VWF defects (VWD type 2) or quantitative VWF deficiencies (VWD type 1 and type 3; partial and virtually complete deficiency, respectively). Partial deficiencies as defined for VWD type 1 may be due to decreased synthesis, impaired secretion, increased clearance or combinations thereof.

The dominant source of circulating VWF is the endothelium, where VWF is synthesized as a single pre-pro-polypeptide chain with a discrete domain structure: D1-D2-D'-D3-A1-A2-A3-D4-B1-B2-B3-C1-C2-CK [4]. After removal of the signal peptide upon entering the endoplasmic reticulum (ER), disulphide bonding within the carboxy-terminal VWF-CK-domains endows formation of pro-VWF dimers. Further processing proceeds within the Golgi, and involves multimerisation of pro-VWF dimers through the formation of intermolecular cystine-bonds within the VWF-D'D3 domains. Proteolytic processing in the trans-Golgi network separates the propeptide (D1-D2 domains) from the mature VWF multimer [5]. Following synthesis in endothelial cells, VWF and its propeptide may be stored in endothelial-specific storage-organelles, the Weibel-Palade bodies [6]. While stored in these organelles, the multimerisation process may continue, giving rise to VWF molecules that consist of higher molecular weight multimers compared to those that circulate in plasma. These high molecular weight multimers have a higher haemostatic potential, and can be released from the Weibel-Palade bodies into the circulation upon stimulation of endothelial cells by various agonists, for instance after desmopressin treatment [7].

Analysis of the primary VWF sequence predicts the presence of 10 O-linked and 12 N-linked glycosylation sites [8], and biochemical analysis has demonstrated the presence of carbohydrate residues at these sites [9,10]. Initial N-linked carbohydrate structures are coupled to VWF in the early stage of synthesis [11]. Further processing into complex N-linked side-chains proceeds within the Golgi, where also O-linked glycosylation occurs, as well as the attachment of sialyl-groups to both O- and N-linked sugars. VWF is one of the rare plasma proteins for which it has been demonstrated that the N-linked carbohydrates contain ABO-blood group determinants [9,10]. These blood group antigens, however, are not present on the O-linked carbohydrates. Interestingly, the nature of the blood-group determinant strongly influences VWF levels: the average VWF levels are approximately 25 % lower in persons with blood group O than those in non-O individuals [12]. A second indication that the glycosylation profile of VWF is an important determinant of VWF plasma levels is illustrated by the finding that the half-life of endogenous VWF is reduced 2-fold in mice genetically deficient for the sialyl-transferase ST3Gal-IV [13]. Moreover, in a patient group referred to the hospital for real

or suspected bleeding disorder, reduced ST3Gal-IV-mediated sialylation was found to be associated with reduced VWF plasma levels [13].

As for the O-linked carbohydrates, no data have been reported about their correlation with VWF plasma levels, apart from one report by Stoddart and colleagues, who demonstrated that recombinant VWF lacking O-linked carbohydrates has a reduced half-life when administered in rats [14]. In the 1980's, studies have been performed regarding the analysis of O-linked carbohydrate structures using VWF purified from therapeutic preparations. Initial analysis revealed some heterogeneity of the O-linked carbohydrate moiety [15], whereas more detailed studies using a combination of methylation studies and NMR-spectroscopy demonstrated that the majority (at least 70 %) of O-linked carbohydrates are composed of the sialylated tumor-associated T-antigen [16]. This structure consists of the disaccharide galactose(β1-3)N-acetylgalactosamine and is sialylated through capping with two N-acetylneuramic acid residues (i.e. NeuAc(α2-3)Gal(β1-3)[NeuAc(α2-6)]GalNAc).

To investigate a potential relation between the occurrence of this glycan structure on circulating VWF and VWF levels, we have designed an assay in which VWF-linked carbohydrates are de-sialylated using neuraminidase, allowing subsequent detection of the remaining T-antigen structure using the lectin Peanut-Agglutinin (PNA). We observed an inverse non-linear association between PNA-binding and VWF plasma levels, which was most pronounced in patients suffering from liver cirrhosis and type 1 VWD.

Materials and Methods

Plasma samples

Plasma from healthy volunteers (n=111) and patients (n=66) was collected in 3.1 % citrate using a Vacutainer system. These patient samples were randomly provided in an anonymised manner by our diagnostic laboratory. Fifty-four patients in stable condition with biopsy-proven liver cirrhosis of various etiology (including alcohol abuse, viral hepatitis, autoimmune hepatitis, primary biliary cirrhosis and cryptogenic cirrhosis) were included in this study [17]. The patients were classified according to Pugh's modification of the Child's classification [18]. Nineteen patients with Child's A cirrhosis, 17 patients with Child's B cirrhosis and 18 with Child's C cirrhosis were studied. VWD type 1 patients (n=32) were classified as VWD type 1 on the basis of bleeding symptoms and VWF-related laboratory criteria (antigen, ristocetin cofactor-activity, and/or multimeric pattern). For some VWD type 1 patients, the underlying genetic defect has been determined (Table 1). Plasma samples of unaffected family members (n=15) were included as control. Other patients (VWD type 2A and 2N, haemophilia A and primary platelet function disorder) were diagnosed using the institutional criteria for these diseases, and anonymised samples were provided for the present study. Treatment with 1-deamino-8-D-arginine vasopressin (DDAVP) was given as part of routine patient care. For normal pooled plasma (NPP), platelet-poor plasma from 40 healthy individuals was pooled and stored in aliquots at -80°C. All patients gave informed consent for the sampling of blood for scientific purposes, per the Declaration of Helsinki. Approval was obtained from the institutional review boards of the University Medical Center Utrecht

Table 1: Genetic defects of VWD type 1 patients

Patient	Mutation	VWF-antigen (U/ml)	Propeptide/VWF ratio (nM/nM)	PNA-binding (% of NPP)
F1-1/2	R854Q	0.37	0.24	150
F1-1/3*	R854Q	0.30	0.21	153
F3-2/1*	R1266L/R1315H	0.28	0.64	269
F3-2/3	R1266L/R1315H	0.10	0.82	361
F3-2/4	R1266L/R1315H	0.18	0.68	274
F4-3/2*	R1342C	0.21	0.27	167
F6-4/2*	G2518S	0.51	0.36	135
F8-5/1*	IVS21 splice	0.30	0.18	164
F8-5/4	IVS21 splice	0.28	0.26	117
F8-5/7	IVS21 splice	0.21	0.24	155
F9-6/3*	L2207P	0.25	0.19	120
F9-6/6	L2207P	0.20	0.28	130
F10-7/1	G2441C	0.20	0.38	141
F10-7/2*	G2441C	0.24	0.35	153
F10-7/3	G2441C	0.24	0.43	155
F10-7/4	G2441C	0.24	0.46	167
LUMC-1*	C2693Y	0.42	0.28	191
LUMC-2	C2693Y	0.44	0.27	189
LUMC-3**	C1149R	0.22	0.72	245
LUMC-4**	C1149R	0.35	0.52	127

*The mutations in these patients have been identified within the European multicenter study "Molecular and Clinical Markers for the Diagnosis and Management of type 1 VWD (MCMDM-1VWD) and have previously been described by Goodeve *et al*.[19]: F1-1/3 = P5F1II3; F3-2/1 = P5F3II2; F4-3/2 = P5F4II1; F6-4/2 = P5F6II3; F8-5/1 = P5F8I3; F9-6/3 = P5F9II2; F10-7/2 = P5F10II1; LUMC-1 = P6F16II1

**LUMC-3 and LUMC-4 have previously been reported as IV-1 and IV-2 respectively, by Eikenboom *et al*.[20]

(Utrecht, the Netherlands), Erasmus Medical Center (Rotterdam, the Netherlands), Leiden University Medical Center (Leiden, the Netherlands), Medical University of Lille (Lille, France) and Centre Hospitalier Universitaire (Nantes, France).

Proteins and antibodies

Polyclonal antibodies against VWF, horseradish peroxidase (HRP)-conjugated antibodies against VWF and HRP-conjugated streptavidin were obtained from DakoCytomation (Glostrup, Denmark). Recombinant llama-derived antibody fragment directed against the A1 domain of VWF (control nanobody) and in-house polyclonal antibodies against VWF D'-D3 domains have been described previously [19,20]. Arthobacter ureafaciens-derived neuraminidase was purchased from Calbiochem (San Diego, CA). Avidin/Biotin blocking kit, biotinylated PNA (btPNA), GalNAc and galactose were obtained from Vector Laboratories (Burlingame, CA). Recombinant O-glycosidase, GlcNAc and glucose were from Sigma (St. Louis, MO), while Gal(β1-3)GalNAc was obtained from Dextra Laboratories (Berkshire, UK).

VWF

Plasma-derived (pd)-VWF was purified from Haemate P (250 IU, Behringwerke, Marburg, Germany) as described [21]. Expression and purification of recombinant wild type (wt)-VWF and VWF-D'-D3 has been described previously [20,22]. cDNA encoding VWF lacking the propeptide (D1-D2 domains) was constructed by fusion of the sequences encoding the VWF signal-peptide (amino acids 1-22) and mature VWF (amino acids 764-2813) using standard molecular-biological techniques. The sequence of the resulting VWF-delta-pro product was verified and the construct was subsequently cloned into the pNUT-vector [22]. cDNAs encoding A1-domain residues 1238-1494 and 1260-1481 (A1/1238-1494 and A1/1260-1481) were obtained by polymerase chain reaction using wt-VWF as a template. PCR-products were sequenced and cloned into a pNUT-expression vector encoding a C-terminal 6-histidine tag [19]. Expression of VWF-delta-pro, A1/1238-1494 and A1/1260-1481 in serum-free medium using baby hamster kidney cells was performed as described for wt-VWF [22].

VWF and propeptide antigen assays

VWF antigen levels were quantified as described before [23]. Propeptide antigen levels were determined using in-house polyclonal rabbit antibodies raised against purified recombinant propeptide. In brief, protein G-Sepharose purified polyclonal antibodies (5 µg/ml) were coated in microtiter wells (Maxisorb, Nunc, Roskilde, Denmark) overnight at 4°C. After washing with phosphate-buffered saline (PBS)/0.1 % (v/v) Tween-20, wells were blocked with the same buffer supplemented with 1 % (w/v) bovine serum albumin and 0.2 M EDTA (dilution-buffer) for 1 h at 37°C. Wells were washed three times with wash-buffer and incubated with plasma samples for 1 h at 37°C. Serial dilutions of plasma samples were prepared using dilution-buffer. After washing 3 times with wash-buffer, plates were incubated with HRP-conjugated polyclonal anti-propeptide antibodies (1 µg/ml) in dilution-buffer for 1 h at 37°C. Plates were washed three times and bound propeptide was detected by measuring HRP-activity using o-phenylenediamine (OPD; Merck, Darmstadt, Germany) as a substrate. NPP was used as a reference in both VWF and propeptide antigen assays.

Immunosorbent assay for VWF-linked T-antigen

Microtiter wells (Maxisorb, Nunc, Roskilde, Denmark) were coated with polyconal anti-VWF antibodies (3 µg/ml) for 2 h at room temperature. Wells were washed three times with PBS/0.1 % (v/v) Tween-20, and incubated with samples for 1 h at 37°C. Samples (either plasma or purified proteins) were diluted in PBS to reach a VWF antigen concentration between 1 and 10 mU/ml, unless stated otherwise. After washing with PBS/0.1 % (v/v) Tween-20, wells were subsequently incubated with neuraminidase (5 mU/ml) in PBS/1 mM $CaCl_2$ overnight at 37°C. Wells were then blocked using the avidin/biotin blocking kit according to the manufacturers' instructions. After washing with PBS/0.1 % (v/v) Tween-20, btPNA (5 µg/ml) was added in PBS/0.1 mM $CaCl_2$ for 1 h at 37°C. Finally, wells were incubated with HRP-conjugated streptavidin. Bound btPNA was detected by measuring HRP-activity using OPD as a substrate. PNA-binding was quantified by calculating the relative slopes of the initial linear parts of the curves. NPP was used as a reference and the slope for NPP was referred to as 100 %. In control experiments, specificity was tested using VWF-deficient plasma, which revealed no PNA-binding in the absence of VWF (not shown). To determine the intra-experiment variation of the assay, 10 randomly

chosen wells were incubated with one sample (healthy volunteer #12). This sample was also tested in 10 different experiments to determine inter-experiment variation. These tests revealed that the intra-experiment variation was 10 % and the inter-experiment variation was 16 %. Throughout the manuscript, PNA-binding refers to neuraminidase-treated samples, unless stated otherwise. In none of the samples tested, PNA-binding was detected when neuraminidase-incubation was omitted.

Data analysis and statistics

Analysis of data were performed using GraphPad Prism program (Graphpad Prism version 4.0 for Windows, GraphPad Software, San Diego, CA). All data are expressed as mean with SD. To address statistical differences, data were analyzed using Student's unpaired two-tailed t-test, with the Welch correction being performed when necessary. *P* values below 0.05 were considered significant.

Results

An immunosorbent assay to detect O-linked sugars on VWF

It has previously been shown that O-linked sugars on VWF primarily consist of the sialylated T-antigen [16]. To detect the presence of this glycan on circulating VWF, a PNA-based immunosorbent assay was developed, in which three main steps can be distinguished: (1) diluted samples are applied to microtiter-wells coated with anti-VWF antibodies; (2) bound VWF is de-sialylated via incubation with neuraminidase; (3) btPNA is added, and bound btPNA is detected using streptavidine-coupled peroxidase. To optimize this assay, we first determined which amount of neuraminidase is needed to remove sialyl-groups from VWF. Various concentrations of neuraminidase (0-4 mU/ml) were added to a fixed amount of antibody-bound pd-VWF (50 mU/ml). No binding of btPNA could be detected in the absence of neuraminidase (not shown), consistent with the fact that PNA only binds to de-sialylated Gal(β1-3)GalNAc. Increasing concentrations of neuraminidase progressively improved btPNA-binding, and near maximal desialylation occurred at 2.5 mU/ml neuraminidase. As for btPNA, a dose-dependent binding could be observed when added to neuraminidase-treated VWF (not shown). Half-maximal binding was observed at 1.5±0.2 µg/ml. Given these data, we decided to use 5 mU/ml neuraminidase and 5 µg/ml btPNA throughout the study.

Specificity of PNA-binding to VWF

The specificity of the assay was then tested in two types of experiments. First, binding of btPNA (5 µg/ml) to neuraminidase-treated VWF (50 mU/ml) was assessed in the presence of various carbohydrate structures. Binding of btPNA to VWF was unaffected in the presence of glucose, GlcNAc, GalNAc or galactose (IC_{50} at >1.0 mM; Figure 1A), even when concentrations up to 10 mM were used (not shown). In contrast, btPNA-binding was efficiently inhibited in the presence of the T-antigen structure: Gal(β1-3)GalNAc (IC_{50} 0.3 mM; Figure 1A). In an alternative approach, the specificity of binding was examined using a number of recombinant VWF variants, including recombinant wt-VWF and VWF-delta-pro

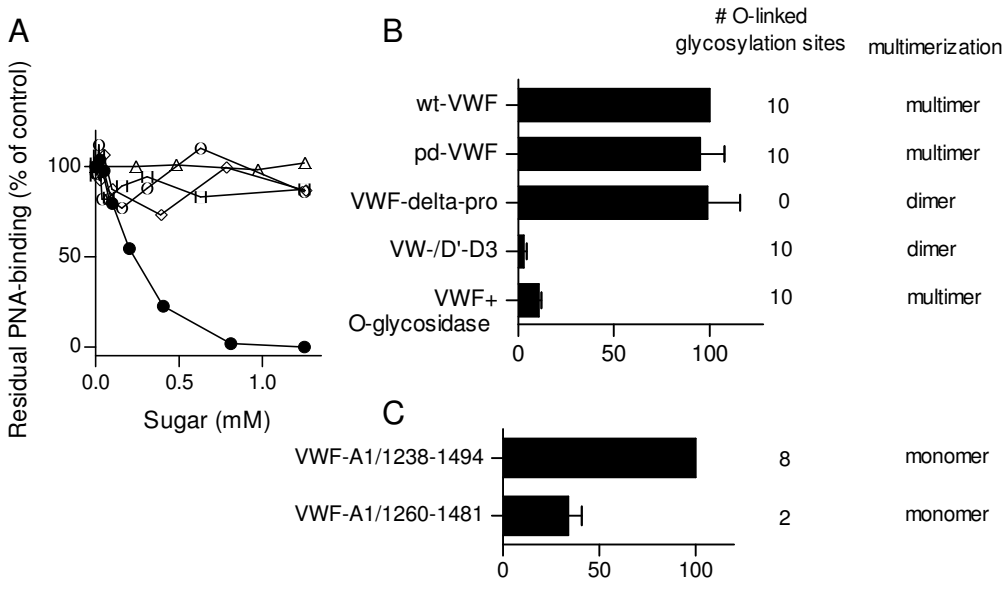

Figure 1. Specificity of PNA-binding to VWF
A: Microtiter wells coated with polyclonal anti-VWF antibodies (3 μg/ml) were incubated with plasma that was diluted in PBS to reach a VWF antigen concentration of 50 mU/ml. After catching VWF, wells were incubated with neuraminidase (5 mU/ml) in PBS/1 mM $CaCl_2$ overnight at 37°C. Subsequently, wells were incubated for 1 h at 37°C with btPNA (5 μg/ml) in the presence or absence of various carbohydrate structures (0-1 mM): open squares: glucose; open circles: galactose; open triangles: GalNAc; open diamonds: GlcNAc; closed circles: Gal(β1-3)GalNAc. After washing, HRP-conjugated streptavidin was added to the wells, and bound btPNA was detected by measuring HRP-activity using OPD as a substrate. Plotted is residual PNA-binding versus sugar concentration. 100 % refers to PNA-binding in the absence of competitors. **B**: Microtiter wells coated with polyclonal anti-VWF antibodies (3 μg/ml) were incubated with equimolar concentrations of pd-VWF, wt-VWF, VWF-delta-pro or VWF-D'-D3 (7.5 nM based on monomer concentration). Where indicated, pd-VWF was incubated with recombinant O-glycosidase (12.5 mU/ml) for 3 h at 37°C following neuraminidase incubation. PNA-binding was determined as described for panel A. Plotted is relative PNA-binding using wt-VWF as a reference (100 %). **C**: Microtiter wells coated with a recombinant anti-VWF A1 antibody were incubated with equimolar concentrations of A1/1260-1481 or A1/1238-1494 (7.5 nM). PNA-binding was determined as described for A. Plotted is relative PNA-binding using A1/1238-1494 as a reference (100 %).

(representing fully multimerized and dimeric VWF, respectively). Both recombinant wt-VWF and VWF-delta-pro were similar to pd-VWF in their ability to bind btPNA (Figure 1B). Also, no difference in PNA-binding was observed for VWF preparations enriched in either high or low molecular weight multimers (not shown), indicating that btPNA-binding is independent of the extent of multimerisation. In addition, we observed little, if any (<5 % compared to wt-VWF) binding of btPNA to VWF-D'-D3, a dimeric construct that contains 3 N-linked but no O-linked carbohydrates. Moreover, PNA-binding was reduced by >85 % upon removal of O-linked glycans via incubation with O-glycosidase (Figure 1B). We further compared two distinct VWF-A1 domain constructs, encompassing residues 1238-1494 and 1260-1481,

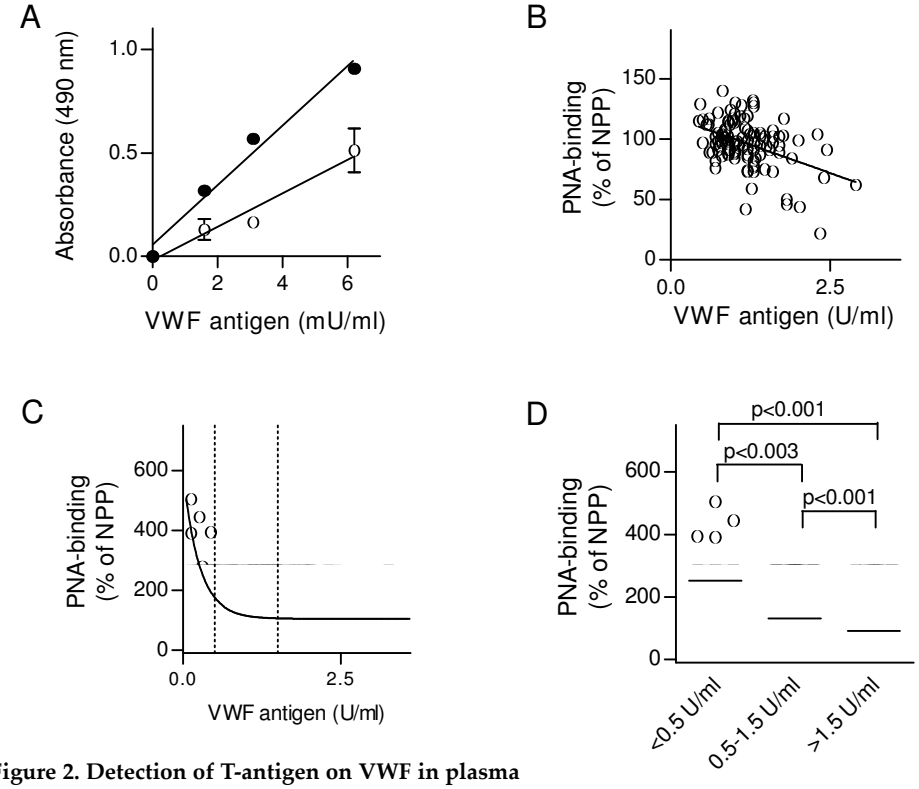

Figure 2. Detection of T-antigen on VWF in plasma

Panel A: Serial dilutions of plasma were added to microtiter wells coated with anti-VWF antibodies, and PNA-binding was determined as described. Plotted is absorbance at 490 nm versus concentration of VWF in diluted samples for NPP (open circles) and a representative sample (closed circles). The slope found for NPP was set at 100%. The slope for this particular sample was calculated to be 177 %. Relative PNA-binding was determined for plasma samples from healthy volunteers (n=111; **panel B**) and samples that were randomly provided by our diagnostic laboratory (n=66; **panel C**). For panel C, the dotted lines indicate VWF values of 0.5 and 1.5 U/ml. The drawn solid lines were obtained by fitting the data in a model for linear regression (Pearson-rank=-0.43; panel B) or a model describing a single exponential decay (r2=0.57; panel C). **Panel D:** Data shown in panel C were subdivided into three groups based on VWF antigen levels that were below 0.5 U/ml (n=15), between 0.5 and 1.5 U/ml (n=32) and above 1.5 U/ml (n=19). Data represent the mean of two independent determinations, and variance between measurements was =16 %. Differences between groups were tested with the unpaired two-tailed t-test, with the Welch correction applied when necessary.

respectively. These constructs comprise 8 and 2 Ser/Thr residues that are known to be O-glycosylated in pd-VWF, respectively (Figure 1C). To analyse btPNA-binding in these experiments, proteins were immobilized using a recombinant llama-derived antibody fragment directed against the A1-domain instead of polyclonal anti-VWF antibodies. Binding of btPNA to the short A1/1260-1481 fragment was decreased by 66±7 % compared to the long A1/1238-1494 fragment (Figure 1C). Taken together, these data indicate that PNA-binding is (1) specific to the de-sialylated O-linked T-antigen structure on VWF, (2) dependent on the number of O-linked glycans and (3) independent of the extent of multimerisation.

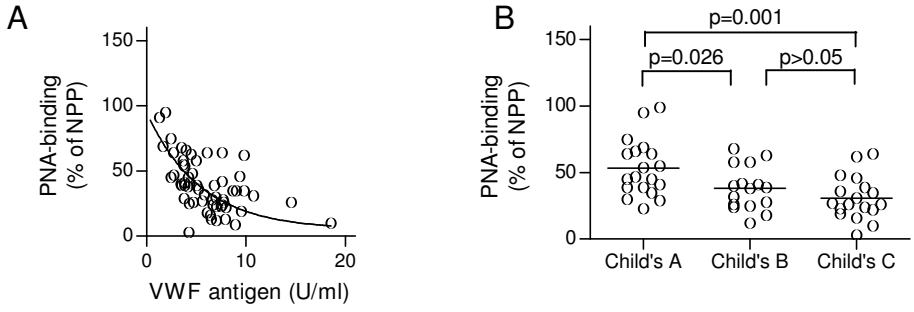

Figure 3. Liver cirrhosis is associated with reduced PNA-binding
Panel A: PNA-binding to VWF was determined in plasma samples of 54 patients diagnosed with liver cirrhosis (see Patients, Materials and Methods section). Plotted is relative PNA-binding versus VWF antigen levels. The drawn line was obtained by fitting the data in a model describing a single exponential decay (r2=0.39).
Panel B: Data shown in panel A were subdivided according to Pugh's modification of the Child's classification of patients. Data represent the mean of two independent determinations, and variance between measurements was =16 %. Differences between groups were tested with the unpaired two-tailed t-test, with the Welch correction applied when necessary.

Association between PNA-binding and VWF plasma levels

The PNA-based assay was subsequently used to determine VWF molecules containing O-linked Gal(β1-3)GalNAc structures in plasma samples of healthy volunteers (n=111). The average VWF antigen level in this group was 1.2±0.5 U/ml. As expected, VWF levels were lower in blood group O-individuals compared to non-O individuals (1.0±0.3 U/ml versus 1.3±0.5 U/ml ($p<0.001$) for O- (n=38) and non-O (n=73) individuals, respectively). PNA-binding was quantified by calculating the relative slopes of the initial linear parts of the curve, and the slope for NPP was referred to as 100% (Figure 2A). Plotting PNA-binding versus VWF antigen levels revealed an inverse relationship between both parameters (Pearson-rank= -0.43; $p<0.0001$; Figure 2B). PNA-binding was higher in persons with blood group O than in non-O individuals (104.5±15.9 % versus 92.6±20.3 % ($p<0.002$), respectively). A similar analysis was also performed in a series of plasma samples that were obtained via the diagnostic laboratory of our institute (n=66). In these samples, VWF levels varied to a larger extent compared to the healthy volunteers: 0.13-4.1 U/ml versus 0.44-2.9 U/ml, respectively. Again, an inverse relationship was observed between PNA-binding and VWF levels (Figure 2C), the data of which were best fitted using an equation describing a single exponential decay ($r^2=0.57$). This relationship became more apparent by arbitrarily dividing the samples into three subgroups: (1) increased VWF levels (>1.5 U/ml: mean±SD=2.2±0.7 U/ml, n=19); (2) intermediate VWF levels (between 0.5 and 1.5 U/ml: mean±SD=1.1±0.3 U/ml, n=32) and (3) reduced VWF levels (<0.5 U/ml: mean±SD=0.3±0.1 U/ml, n=15). PNA-binding in the group with reduced VWF levels (252±125 %) was higher than in the other two groups ($p=0.0024$ and $p=0.0002$ for the intermediate and high group, respectively; Figure 2D). Moreover, a significant difference was found between the intermediate and high VWF-level groups (131±36 % and 92±40 %,

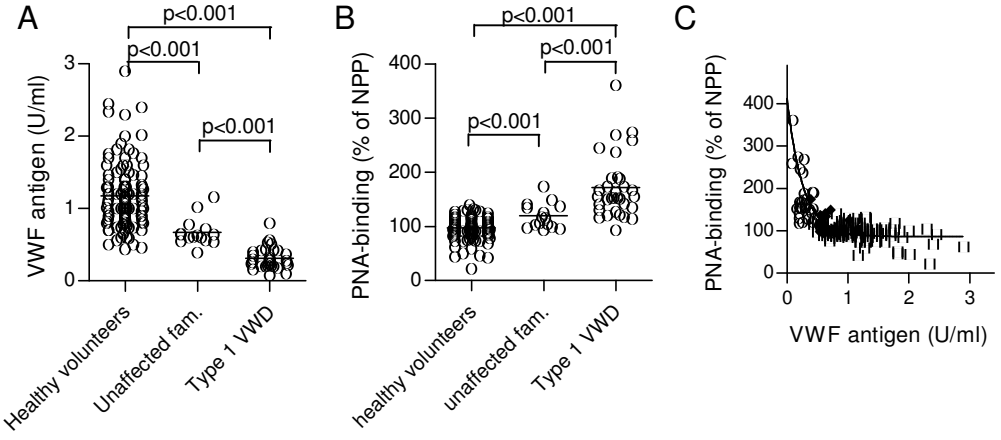

Figure 4. VWD type 1 is associated with increased PNA-binding
VWF antigen levels (**panel A**) and PNA-binding (**panel B**) were determined in plasma samples of healthy volunteers (n=111), unaffected family members (n=15) and patients diagnosed with VWD type 1 (n=32). Data represent the mean of two independent determinations, and variance between measurements was =12 % (VWF antigen levels) and =16 % (PNA-binding). In **panel C**, PNA-binding versus VWF antigen levels is depicted: Depicted are healthy volunteers (open squares), unaffected family members (closed triangles) and VWD type 1 patients (open circles).

respectively; p=0.0006; Figure 2D). Apparently, there seems to be an association between VWF levels and the presence of the sialylated O-linked Gal(β1-3)GalNAc structure on VWF.

Reduced PNA-binding to VWF in patients with liver cirrhosis

To investigate the relationship between VWF plasma levels and the presence of Gal(β1-3)GalNAc structures in more detail, we examined plasmas of patients with liver cirrhosis and elevated VWF levels. This patient group has been described previously [17], and three categories of patients can be distinguished according to the Pugh's modification of the Child classification [18]. The VWF levels were 4.5±2.3 U/ml (n=19), 5.5±2.8 U/ml (n=17) and 7.8±3.4 U/ml (n=18) for Child's A, Child's B and Child's C classified patients respectively. When tested for PNA-binding, we could again observe an inverse nonlinear relationship between VWF and PNA-binding (Figure 3A). In addition, a correlation between the severity of the disease and relative PNA-binding was observed when the patients were subdivided according to the Child classification (Figure 3B). The more severe the disease, the lower the PNA-reactivity towards VWF: 53±21 %, 41±20 % and 31±16 % for Child's A, Child's B and Child's C classified patients, respectively. These data indicate that increased VWF levels in liver cirrhosis patients are characterized by a suboptimal glycosylation pattern with O-linked Gal(β1-3)GalNAc.

Increased PNA-binding in patients with VWD type 1

To study binding of PNA to VWF present in plasma with reduced VWF levels, we collected samples from patients with VWD type 1 (n=32), the genetic defect of whom was established in 20 patients (Table 1). As a control, plasma from healthy volunteers (n=111) and non-affected type 1 family members (n=15) were used. The average VWF antigen levels were 1.2±0.5 U/ml,

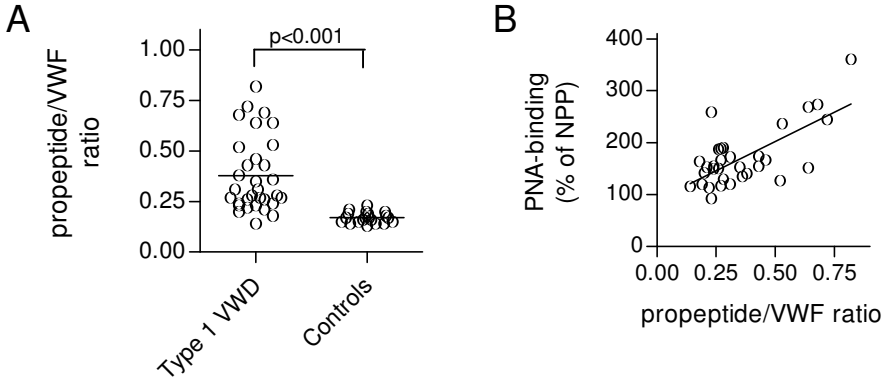

Figure 5. PNA-binding correlates with propeptide/VWF ratios-
Panel A: VWF propeptide and mature VWF antigen levels were determined for healthy volunteers (n=24) and VWD type 1 patients (n=32) depicted in Figure 4, and the molar ratio propeptide over VWF was calculated as described[59]. Difference between both groups was tested using the unpaired two-tailed t-test with the Welch correction applied. **Panel B:** Values for relative PNA-binding obtained for type 1 VWD patients were plotted versus the corresponding propeptide/VWF ratios. The drawn line was obtained after linear regression analysis. Correlation was found to be significant (Spearman-rank=0.50; p=0.004).

0.7±0.2 U/ml and 0.3±0.2 U/ml for healthy volunteers, non-affected family members and type 1 patients, respectively (Figure 4A). PNA-binding was significantly increased in non-affected family members (119±24 %; p<0.0001), and to a larger extent in samples from VWD type 1 patients (171±58 %; p<0.0001; Figure 4B) when compared to healthy volunteers (96.7±20.7 %). Thus, low levels of VWF present in VWF type 1 patients and (to a lesser extent) their unaffected family members are associated with increased PNA-binding upon neuraminidase-incubation, suggesting the presence of a surplus of O-linked sialylated T-antigen on their VWF molecules.

Association between PNA-binding and propeptide/VWF ratio in type 1 VWD
Shortage of circulating VWF may originate from abnormalities in the balance between production and clearance. A surrogate marker for VWF clearance is represented by propeptide/VWF ratios, which are indeed increased in several VWD type 1 patients who have an unusual short VWF survival [26]. As for VWD type 1 patients in the present study, the average propeptide/VWF ratio was increased 2-fold compared to controls (0.38±0.18 and 0.17±0.03 for patients and controls, respectively; p<0.0001; Figure 5A), suggesting that VWF is cleared more rapidly, at least in part of the patients. When divided in quartiles with respect to propeptide/VWF ratios, patients with the lowest ratios (ranging from 0.14 to 0.23; n=8) had mean VWF levels of 0.45±0.20 U/ml and PNA-binding was 145±51 %. For the quartile with the highest ratios (0.46-0.82; n=8) values were 0.22±0.08 U/ml and 229±77 % for VWF antigen levels and PNA-binding, respectively. For both parameters, these values were significantly different between both quartiles (p<0.03). We then plotted the extent of PNA-binding versus propeptide/VWF ratios for all patients (Figure 5B). Interestingly, a linear correlation between

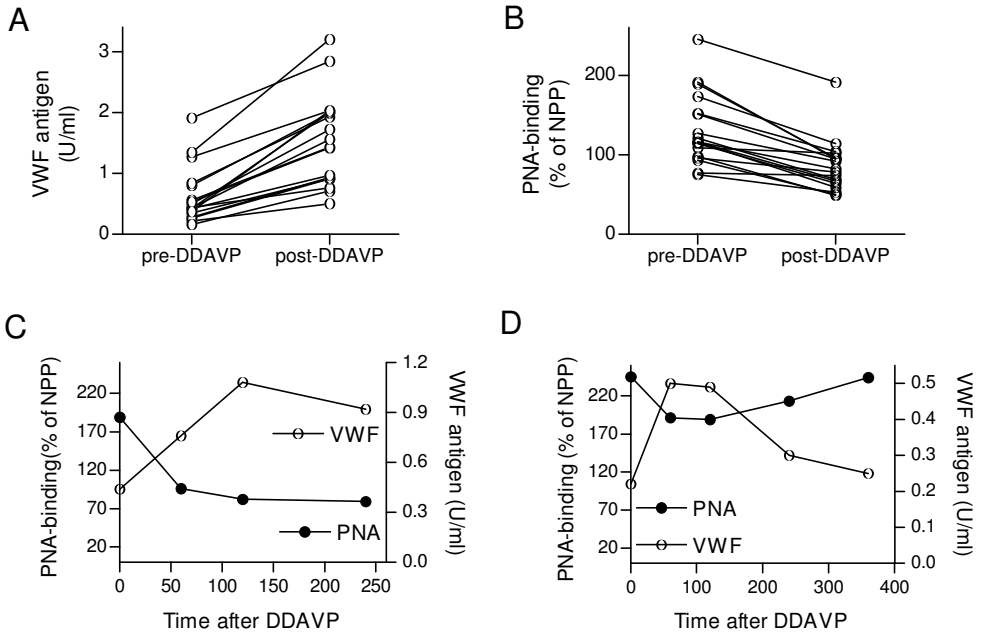

Figure 6. Reduced PNA-binding to post-DDAVP VWF
For a selection (n=11) of the VWD type 1 patients (see Figure 4) as well as VWD type 2 A (n=2), VWD type 2N (n=1) haemophilia A (n=1) and storage-pool disease (n=3) patients, VWF antigen levels (**Panel A**) and PNA-binding (**Panel B**) were determined in plasma samples that were taken prior or 60 min after DDAVP treatment. Mean VWF levels were 0.62±0.46 U/ml and 1.55±0.74 U/ml pre- and post DDAVP, respectively (p=0.0001). Mean PNA-binding was 131±45 % and 85±33 % (p=0.0013). For two patients, changes in VWF antigen levels (**panels C and D**: right axes) and PNA-binding (**panels C and D**: left axes) were followed in time.

PNA-binding and propeptide/VWF ratios was observed (Spearman-rank=0.50; p=0.004). Apparently, an increased extent of glycosylation with O-linked T-antigen is associated with high propeptide/VWF ratios. This may point to a relationship between increased numbers of sialylated Gal(β1-3)GalNAc structures on VWF and reduced survival of VWF.

Dissimilar PNA-binding to pre- and post-DDAVP VWF

Part of VWF that is synthesized in endothelial cells remains stored intracellularly in Weibel-Palade bodies. These Weibel-Palade bodies release their content upon DDAVP treatment, which increases VWF levels in plasma in a temporary manner. This treatment strategy is applied not only in type 1 VWD but also in other disorders, such as mild haemophilia A and primary platelet function disorders. To investigate the effect of DDAVP-treatment on the extent of glycosylation of VWF with O-linked sialylated T-antigen, we analyzed pre- and post-DDAVP samples of 18 patients who had received DDAVP as part of routine patient care. These included a subset of the type 1 VWD patients presented in Figure 5 (n=11), VWD type 2A (n=2), VWD type 2N (n=1), Haemophilia A (n=1) and primary platelet function disorder (n=3). VWF levels raised in all patients treated with DDAVP (Figure 6A), with mean VWF

levels being 0.6±0.5 U/ml before treatment, which increased up to 1.6±0.7 U/ml 60 min after DDAVP-treatment (p=0.0001). In two of the samples tested, the extent of PNA-binding to VWF after neuraminidase-incubation was slightly, if any (<5%) reduced in the post-DDAVP sample. Surprisingly, in all of the other samples tested (16 out of 18), a significant drop in PNA-binding was observed, which varied between 20 and 50 % (Figure 6B). For two patients, samples of additional time-points were available. In one patient, VWF levels remained increased up to 4 hours, with PNA-binding being reduced during this time-course (Figure 6C). For a second patient, a short-lasting increase in VWF was observed as antigen returned to base-line levels at 4 hours after DDAVP-treatment. This indicates that the half-life of VWF of this patient is relatively short, a possibility that is supported by an increased propeptide/VWF ratio that was calculated to be 0.72 (patient LUMC-3, see Table 1). As for PNA-binding, an initial drop was followed by a rapid restoration, in a manner that inversely mimicked changes in antigen levels (Figure 6D). In conclusion, these data indicate that O-linked glycosylation of VWF stored in endothelial Weibel-Palade bodies differs from VWF that circulates in plasma.

Discussion

Glycosylation has been reported to contribute to correct folding, intracellular routing and/or optimal functionality of proteins [27-29]. In addition, changes in the glycosylation profile may affect the survival of proteins in the circulation [30]. One particular example hereof is provided by VWF [31]. In animal models as well as for humans, it has been shown that the VWF glycosylation profile is an important determinant of its plasma levels and survival [13,14, 23, 32]. At present, little is known concerning the contribution of O-linked carbohydrates in this regard. In search for a potential relationship between O-linked glycans and VWF levels, we took advantage of the previous finding that the O-linked carbohydrate structure primarily consists of sialylated T-antigen [16]. Upon de-sialylation, the remaining T-antigen structure (also known as the Thomsen-Friedenreich antigen) on VWF is recognized by the lectin PNA [33], and this lectin can therefore be used as a tool to analyse the presence of this carbohydrate structure. The specificity of our immunosorbent-based assay was illustrated by: (i) the lack of PNA-binding when neuraminidase-treatment was omitted (not shown), (ii) the efficient inhibition of PNA-binding by the T-antigen structure Gal(β1-3)GalNAc (IC_{50} ~0.3 mM), whereas other sugar structures proved ineffective (IC_{50} > 10 mM; Figure 1A), (iii) the lack of PNA-binding to a VWF fragment that contains N-linked but no O-linked glycans or to VWF treated with O-glycosidase (Figure 1B). Furthermore, PNA-binding proved to be dependent on the number Ser/Thr residues known to carry O-linked glycans (Figure 1C). Finally, PNA bound similarly to either dimeric VWF or fully multimerized VWF (Figure 1B), showing that the assay is independent of the extent of multimerisation.

Using this assay, we examined a series of 111 samples obtained from healthy individuals. VWF levels varied between 0.5 and 3 U/ml, while PNA-binding varied between

22 and 140% (Figure 2B). Further analysis revealed a statistically significant association between both parameters (Figure 2B). A more pronounced variation in PNA-binding (between 17 % and 505 %) was observed in samples that were randomly provided by our institutional diagnostic laboratory. It should be noted that these samples have been analysed blindly, being unaware of the potential underlying pathology. The fact that these samples are sent to this laboratory for testing of haemostatic parameters may explain the relatively high number of samples with high or low VWF levels. Like for the healthy individuals, we found that the extent of PNA-binding highly associated with VWF levels (Figure 2C), albeit that PNA-binding increased sharply in a number of samples when VWF levels dropped below 0.5 U/ml. These data therefore indicate that the more O-linked sialylated Gal(β1-3)GalNAc structures are present on VWF, the lower the VWF levels seem to be. As such, the O-linked sialylated T-antigen constitutes a novel carbohydrate structure that associates with VWF plasma levels. Of importance, our data do not distinguish whether this structure should be considered as a determinant, or whether it only correlates with VWF antigen levels. Additional studies are required in this respect. Other glycosylation-related elements that have been reported to influence VWF levels include ST3Gal-IV transferase (which adds sialyl-groups to terminal galactose residues), α(1,2)-fucosyltransferase (which adds terminal fucose to glycans, and is encoded by the Secretor-locus) and the ABO-blood group structures, which are added to N-linked carbohydrates [12,13,34].

The variation in PNA-binding in relation to VWF levels was explored in more detail using two distinct patients groups. One group consisted of a heterogeneous but well-characterized patient-cohort with liver cirrhosis and elevated VWF levels (up to 18.6 U/ml; Figure 3 and Lisman *et al* [17]). In this group, high levels of VWF correlated with reduced PNA-binding, suggesting a low occupation with Gal(β1-3)GalNAc. Moreover, this low occupation correlated with the severity of the disease (Figure 3B). It remains to be investigated whether this reduced glycosylation of VWF with sialylated T-antigen results from disease-related impairments in the glycosylation pathway, or that VWF production is too high to allow full glycosylation with this carbohydrate structure. Alternatively, a prolonged survival of the VWF molecules in the circulation due to impaired hepatic clearance may result in prolonged exposure to glycosidases that remove O-linked sugars. However, this option seems less likely since (i) one would expect sialyl-groups to be removed prior to removal of O-linked glycans, and no PNA-binding prior to neuraminidase-treatment could be detected, and (ii) to our knowledge no such glycosidases have been reported to be present in plasma. Another explanation for our observations could be that the sites for O-linked glycosylation are used for linking of other non-T-antigen structures. Such changes in O-linked glycosylation occur for instance in the so-called Tn-syndrome [35]. In this syndrome, there is reduced polypeptide β1-3-galactosyltransferase (T-synthase) activity [35,36]. Consequently, the O-linked carbohydrate structure consists predominantly of the Tn-antigen (GalNAcα1-Ser/Thr) in stead of the T-antigen, and this Tn-antigen is not recognized by PNA. Interestingly, platelet VWF of patients with the Tn-syndrome reacts differently with anti-VWF antibodies when assessed via crossed-immuno-electophoresis [37], underscoring the notion that O-linked glycosylation is an

essential element of VWF biology. Of note, mice genetically deficient for T-synthase indeed lack T-antigen bearing proteins, and these knock-out mice display defective angiogenesis and fatal embryonical haemorrhage [38].

The second group of patients with aberrant VWF levels consisted of VWD type 1 patients in whom antigen levels are reduced (Figure 4A). In opposite to liver cirrhosis patients, we found that VWD type 1 patients were characterized by increased PNA-binding in our assay (up to 361 %). We considered the possibility that VWF molecules could have been hypo-sialylated, making them potentially more susceptible for PNA-binding. However, since we could not observe any PNA-binding in the absence of neuraminidase-incubation in any of the samples tested, this possibility seems rather unlikely. Alternatively, increased PNA-binding may indeed correlate with an increased number of O-linked sialylated Gal(β1-3)GalNAc structures per VWF molecule. This raises of course the question to which residues within the VWF molecule these additional carbohydrate structures are coupled. It is of importance to mention in this regard, that structural requirements for O-linked glycosylation are less well-defined compared to those for N-linked glycosylation. Dependent on the algorithm for prediction that is used, different amino acids may fulfil the criteria as potential target for O-linked glycosylation. Thus, the excess of PNA-binding could be explained by the presence of O-linked Gal(β1-3)GalNAc structures that are coupled to non-predicted glycosylation sites. On the other hand, it can not be excluded that certain conditions allow the addition and exposure of Gal(β1-3)GalNAc structures on N-linked glycans. Another option that could explain increased PNA-binding is that a mutation-associated disturbance of the VWF conformation results in a better exposure of the Gal(β1-3)GalNAc element, allowing more efficient PNA-binding.

A substantial portion of the VWD type 1 patients was characterized by increased propeptide/VWF antigen ratios (Figure 5A). From several studies it has become evident that increased ratios in VWD type 1 patients are associated with a reduced survival of endogenous VWF [39,26]. Since altered glycosylation may affect protein survival, it was of interest to analyse the relationship between PNA-binding and propeptide/VWF ratio. This analysis revealed a linear correlation between both parameters (Figure 5B), pointing to the possibility that increased glycosylation of VWF with O-linked sialylated Gal(β1-3)GalNAc results in decreased survival of VWF in these patients. However, our data do not allow to distinguish between reduced survival originating from the molecular defect itself, increased glycosylation with the Gal(β1-3)GalNAc or a combination of both. On the other hand, the contribution of increased Gal(β1-3)GalNAc coupling would provide an explanation for the observations that increased clearance of VWF has been found in VWD type 1 patients with unrelated genetic defects [22,26,40,41]. We are currently investigating whether or not aberrant glycosylation of VWF with O-linked sialylated Gal(β1-3)GalNAc also occurs in other types of VWD or in other patients with reduced VWF levels.

An often-applied treatment protocol to accomplish a temporary rise in VWF levels involves administration of the desmopressin analogue DDAVP. This treatment provokes release of VWF that is stored in the endothelial Weibel-Palade bodies. The availability of

patient samples before and after DDAVP-treatment allowed us to analyze for the effect of VWF storage on glycosylation. Interestingly, there was a marked decrease (20-50 %) in PNA-binding in 16 out of 18 samples tested (Figure 6B). This was observed not only in VWD type 1 patients, but also in non-VWD patients (haemophilia A, storage pool disease), illustrating that it represents a general effect that is not restricted to VWD. Moreover, it provides an alternative explanation for reduced PNA-binding to VWF from patients with liver cirrhosis. It cannot be excluded that the increase in VWF levels in these patients is related to acute endothelial damage [17], a condition that results in the release of the Weibel-Palade bodies. The differences in O-linked glycosylation of VWF in pre- and post-DDAVP samples indicates that the glycosylation pattern of VWF stored in Weibel-Palade bodies differs from VWF that circulates in plasma. Whether differences in O-linked glycosylation contribute to the intracellular sorting and/or secretion of VWF is a challenging concept, but awaits further studies.

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CHAPTER 3

CHAPTER 3

Cysteine mutations in von Willebrand factor associated with increased clearance

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Abstract

Von Willebrand disease (VWD) is a bleeding disorder caused by the decrease of functional von Willebrand Factor (VWF). Low levels of VWF can result from decreased synthesis, impaired secretion, increased clearance or combinations thereof. Several mutations lead to impaired synthesis or secretion of VWF, however, little is known about the survival of VWF in the circulation. To evaluate the effect of several VWF mutations on VWF clearance. The effect of three cysteine mutations (C1130F, C1149R or C2671Y) on the in vivo survival of VWF was studied in patients carrying these mutations and in a VWF-deficient mice model. In patients carrying these mutations we observed increased propeptide/mature VWF ratios and rapid disappearance of VWF from the circulation after desmopressin treatment. Detailed analysis of in vivo clearance of recombinant VWF in a VWF-deficient mice model revealed a 4-fold increased clearance rate of the mutants. The mutations C1130F, C1149R and C2671Y are each associated with reduced survival of VWF in the circulation. Detailed analysis of the recombinant mutant VWF demonstrated that increased clearance was not due to increased proteolysis by ADAMTS13. We did not identify functional or structural characteristics that the mutant proteins have in common and could be associated with the phenomenon of increased clearance. Cysteine-mutations in VWF may result in reduced in vivo survival. The observation that various mutations are associated with increased in vivo clearance may have major implications for the therapeutic strategies that rely on the rise of endogenous VWF after desmopressin administration.

Introduction

Von Willebrand factor (VWF) is a plasma glycoprotein that plays a dual role in haemostasis. First, VWF forms a complex with coagulation Factor VIII (FVIII), which is required for FVIII survival in vivo. Second, VWF contributes to platelet adhesion and aggregation by acting as a molecular bridge between subendothelial collagen and platelets [1].

VWF is produced in megakaryocytes and endothelial cells, where it is subjected to extensive posttranslational modifications, including proteolytic separation of the precursor molecule in a propeptide and mature VWF. VWF maturation involves C-terminal dimerization and propeptide-dependent N-terminal multimerization via the formation of covalent intermolecular cystine-bonds [1]. The ability of VWF to support platelet adhesion and aggregation increases with multimer size [1]. In the circulation, multimer size is controlled by several mechanisms, including proteolytic cleavage by ADAMTS13 [2,3]. VWF is also subjected to N- and O-linked glycosylation and is one of the rare plasma proteins that carry the blood group antigens A, B and H [4]. The VWF glycosylation profile is one of the determinants of VWF survival in the circulation [5,6]. Besides altered glycosylation, amino acid substitutions in VWF may also affect clearance. Recently, we have used a mouse-model to demonstrate that the mutation R1205H leads to a strongly reduced survival of VWF in the circulation [7].

Defects in the VWF gene result in a bleeding disorder (von Willebrand disease, VWD) with variable penetrance. VWD can be categorized in qualitative VWF defects (type 2) and quantitative VWF deficiency (type 1 and type 3; partial and virtually complete deficiency, respectively) [1]. Most mutations in the human mutation database that are associated with the quantitative deficiencies originate from null alleles (gene deletions, stop codons, frame shifts, and splice mutations), but missense mutations have also been reported. Some of these mutations lead to replacement of cysteine-residues, like C1130F, C1149R and C2671Y [8,9]. Mutations C1130F and C1149R are both located in the VWF D3-domain and are recognized as dominant-negative mutations [8,10]. Patients heterozygous for C1130F and C1149R display a pronounced quantitative VWF deficiency, a prolonged bleeding time and a history of moderate bleeding [8,11,12]. Mutation C2671Y is located towards the carboxyterminus of the molecule in the connective region between the C2- and CK-domains. The mutation has been reported for a single patient who is compound heterozygous for this mutation and a gene deletion. The patient displays a type 3 phenotype with VWF antigen (VWF:Ag) levels of 0.02-0.04 U/ml, and part of the residual circulating VWF protein consists of proteolysed products [8,13].

Analysis of recombinant variants of VWF/C1130F and VWF/C1149R revealed that these mutants lacked high molecular weight multimers, which defect could be corrected upon co-expression with wt-VWF. In contrast, VWF/C2671Y showed a normal multimerization pattern. All three mutations resulted in reduced secretion of VWF due to intracellular retention, and, at least for VWF/C1149R, proteosomal degradation [8,10,12]. However, the plasma VWF-antigen (VWF:Ag) levels found in the patients were much lower than the VWF levels observed in the in vitro expression experiments [12]. This indicates that the intracellular

retention and degradation of the mutants only partly explain the low VWF levels in the patients. In the present study we explored the possibility that modified clearance also contributes to the reduced VWF levels in these patients. By investigating the behaviour of endogenous VWF upon desmopressin treatment of patients as well as the survival of recombinant VWF mutants in an experimental model employing VWF deficient mice, we have indeed obtained evidence that increased clearance contributes to the reduced VWF levels in vivo.

Materials and Methods

Patients, mutations and phenotypic tests

The C1130F and C1149R mutations were originally identified in patients classified as type 1 VWD, characterized by very low VWF:Ag levels (0.10-0.15 IU/ml) [9]. The C2671Y mutation was found in a type 3 VWD patient, compound heterozygous for this mutation and a deletion of the other allele [8]. Plasma was collected on several occasions from seven patients and two unaffected family members (Table 1). An infusion with 1-deamino-8-D-arginine vasopressin (DDAVP; 0.3 mg DDAVP/kg body weight) was performed after informed consent. Blood samples were analysed for VWF:Ag by an immunosorbent assay, VWF ristocetin cofactor activity by aggregometry using fixed human platelets and FVIII activity using a one-stage clotting assay. The concentrations of VWF-propeptide antigen were analysed in an immunosorbent assay as described [14,15]. A plasma pool containing 6.3 nM propeptide and 50 nM VWF (monomer-concentration) was used as a standard.

Mice

The VWF-deficient 16 and wild-type mice were on a C57BL/6J background and were between 8 and 12 weeks old. Housing and experiments were done as recommended by French regulations and the experimental guidelines of the European Community.

Proteins

Recombinant Glycoprotein-Ibα (GpIbα, residues 1-290) was prepared as described [17]. The GpIbα antibody (2D4) was a gift of H. Deckmyn (Kortrijk, Belgium). Botrocetin was from Kordia (Leiden, the Netherlands). Purified recombinant VWF-propeptide was prepared as described [14]. Recombinant B-domain deleted FVIII (Refacto) was from Wyeth. Human collagen type III (catalogue-number C-4407) and bovine albumin (fraction V) were from Sigma. Human albumin (fraction V) was from MP Biochemicals (Irvine, CA USA). Polyclonal antibodies (unlabeled and peroxidase-conjugated) against VWF were from Dako (Glostrup, Denmark).

Table 1: Patient characteristics

VWF	Family and patients*	FVIII:C (IU/ml)	VWF:Ag (IU/ml)	VWF:RCo (IU/ml)	Blood group
C1130F/wt	C Proband	0.19	0.14	<0.20	O
	C Sister	0.2-0.25	0.13-0.16	<0.20	O
	B Proband	0.45	0.13-0.21	<0.20	A
C1149R/wt	III-4	0.18-0.21	0.10-0.21	<0.20	A
	IV-1	0.22-0.29	0.11-0.22	<0.20	A
	IV-2	0.35-0.74	0.15-0.35	<0.20	O
	III-5†	1.17	0.83-0.91	0.93	O
	IV-3†	1.09	0.86-1.33	0.91	A
C2671Y/del	A III-2	0.17	0.10	<0.20	O
Normal range		0.5-2	0.4-2	0.5-2	

* Patients and family members are indicated according to the original articles [11,8,9].
† Unaffected family members.
FVIII:C, FVIII activity; VWF:Ag, VWF antigen; VWF:RCo, VWF ristocetin cofactor activity.

Recombinant VWF

All plasmids were constructed using conventional techniques and all constructs were sequenced before transfection. Plasmids pSVH-VWF encoding wt-VWF, rVWF/C1130F, rVWF/C1149R and rVWF/C2671Y were transiently expressed in 293T human kidney cells 12. These recombinant variants were used to analyse ADAMTS13-mediated proteolysis. Plasmids pNUT-VWF encoding wt-VWF, rVWF/C1130F, rVWF/C1149R and rVWF/C2671Y were stably expressed in baby hamster kidney-cells overexpressing furin for proper removal of the propeptide [7,18,19]. cDNA encoding wt-VWF was subcloned into pcDNA6 for co-transfection to rVWF/C1149R-expression cells, allowing selection using blasticidin to establish stable cell-lines expressing heterozygous wt-VWF-rVWF/C1149R. All recombinant variants were purified from conditioned serum-free medium, and used in the various functional assays and to determine VWF clearance in mice.

ADAMTS13-mediated proteolysis

The pcDNA3.1-ADAMTS13 expression vector (provided by J. Voorberg, CLB research at Sanquin, Amsterdam, The Netherlands) was used for transient expression of recombinant ADAMTS13. Conditioned medium was collected and Pefabloc SC (Roche Diagnostics, Mannheim, Germany) was added to a final concentration of 1 mM. Medium was concentrated ~10 times by centrifugation in 5 mM Tris (pH 8.0) using Macrosep concentrators (cut-off value 100 kDa; Pall Gellman Laboratory, Ann Harbor, USA). ADAMTS13 protease activity was determined using previously described methods and conditions [20,21]. The sensitivity of a fixed concentration of mutant, cotransfected or wt VWF was assessed with a 1:20 dilution of ADAMTS13 in 5 mM Tris (pH 8.0) with 12.5 mM $BaCl_2$ to initiate the reaction. The final concentration of urea in the reaction was 0, 0.1, 0.5, 1.0 or 1.5M. Aliquots were taken after 0, 8 and 24h incubation at 37°C and the reaction was stopped with EDTA. Subsequently, the VWF multimeric structure was analysed on denaturing, non-reducing agarose gels (1.5% (w/v)) 12. In all tests the same batch of concentrated ADAMTS13 was used.

Clearance of purified recombinant VWF in mice

Clearance of recombinant wt-VWF and mutant VWF has been analysed as described [7,16]. Three to six mice were used for each time-point, and each mouse was bled only once. Human VWF:Ag levels were quantified as described [15].

GpIbα binding

Monoclonal anti-GpIbα antibody 2D4 was immobilized in microtiter-wells (Costar, Cambridge MA, USA) in 50 mM $NaHCO_3$ (1.0 µg/ml, overnight at 4°C), which were then blocked for 1h at 37°C with PBS/3% (w/v) bovine albumin/0.1% (v/v) Tween-20). Recombinant GpIbα was added (0.1 mg/ml for 2h at 37°C) and subsequently increasing concentrations of VWF (0-4 nM) were applied in the presence of botrocetin (2 mg/ml) and incubated for 2h at 37°C. After washing, wells were incubated with peroxidase-labeled polyclonal anti-VWF antibodies (1.3 mg/ml for 1h at 37°C), and bound VWF was detected by measuring peroxidase activity using o-phenylenediamine as a substrate.

αIIBβ3-dependent platelet adhesion to immobilized VWF

Perfusion with platelets (shear-rate 1600 s-1) over VWF-coated cover slips was performed as described elsewhere [22]. The amount of platelet-adhesion was evaluated using computer-assisted analysis with OPTIMAS-6.0 software (Dutch-Vision-Systems, Breda, The Netherlands), and was expressed as the percentage of surface-coverage. Perfusions were performed 6-9 times for all variants.

Surface Plasmon Resonance analysis

Several binding assays were performed employing a Biacore2000 system (Biacore AB, Sweden). Binding of propeptide and FVIII to immobilized VWF or its mutants was investigated as described [7], exept that recombinant B-domain deleted FVIII (Refacto) was used instead of FVIII light chain. Binding of VWF or its derivatives to immobilized collagen type III was performed as described [15].

Data analysis and statistics

Analysis of protein-interaction assays and clearance data obtained from mouse-experiments was performed using the GraphPad Prism program (GraphPad Prism version 4.0 for Windows, GraphPad Software, San Diego, CA, USA) as described [7]. Clearance data obtained from the patients, after subtraction of the basal VWF and propeptide level from the post-DDAVP values, were fitted to the monoexponential equation $C_t = Ae^{-\alpha t}$ to obtain a. C_t refers to the plasma concentration of VWF or propeptide at time-point t after DDAVP infusion, and the apparent half-life was calculated from the equation $t1/2 = \ln 2/\alpha$. Statistical analyses were performed by the Student's unpaired t-test using the GraphPad InStat program (GraphPad InStat version 3.00 for Windows).

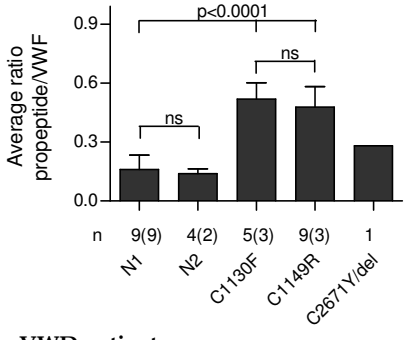

Figure 1. Propeptide/VWF ratio in VWD patients
The average ratio of propeptide over mature VWF for normal controls (N1), unaffected family members from the family with the C1149R mutation (N2), and patients harboring the C1130F, C1149R or C2671Y/del mutation at basal level are depicted. The number of measurements (n) is indicated, with the number of patients in brackets. Differences between groups were tested with the unpaired two-tailed t-test. Data represent mean±S.D., and data of N1 were obtained in a previous study [23]. NS, not significant

Results

Increased VWF-propeptide/mature VWF ratio

VWF is secreted simultaneously with its propeptide at equimolar concentrations and they circulate at a distinct ratio [14,23]. The patients in this study display VWF:Ag levels that are well below the normal range (Table 1). In contrast, the VWF-propeptide levels were less reduced, resulting in an increased ratio of propeptide over VWF:Ag (Figure 1). These ratios were increased three-fold for patients with the mutations C1130F and C1149R compared to the normal population. The ratio was also increased in the compound heterozygous patient (C2671Y/deletion) (Figure 1). These data suggest a reduced half-life of VWF, or, alternatively, a prolonged half-life of propeptide.

Increased clearance of VWF upon DDAVP treatment

To test whether the modified ratios result from a change in half-life of propeptide or mature VWF, plasma levels of VWF and propeptide were monitored at different time-points after DDAVP infusion. Healthy subjects and patients showed a rapid increase of the propeptide level, which was followed by a monophasic decay (Figure 2A). The half-life of propeptide in patients with C1130F or C1149R was similar to healthy subjects, whereas the half-life of propeptide in the patient with C2671Y was somewhat longer (Figure 2A, Table 2). VWF showed a similar rapid increase followed by a monophasic decay (Figure 2B). In the patient group, VWF was cleared much quicker than in the group of healthy subjects. Indeed, the estimated half-life of VWF in the patients with mutations C1130F, C1149R and C2671Y was reduced 4-5 fold (Figure 2B, Table 2). It should be noted that the estimated half-lives were based on a limited number of time points, which prevents accurate calculation of the half-lives. Nevertheless, these data strongly indicate that the mutations C1130F, C1149R and C2671Y are associated with an increased clearance of mature VWF.

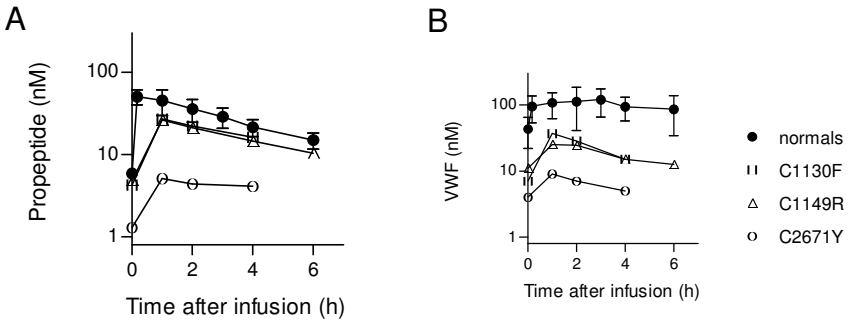

Figure. 2. Survival of endogenous propeptide and VWF after DDAVP administration
Blood was collected before and after DDAVP infusion (0.3 mg/kg body weight). Samples were analyzed for propeptide (**Panel A**) and VWF (**Panel B**) levels. Shown are the average±S.D. for controls (n=9) and data from single experiments in patients. Data of the normal controls were obtained in a previous study [23]. The apparent half-lives are listed in Table 2.

Table 2. Apparent half-lives of propeptide and VWF after DDAVP treatment

VWF	$t_{1/2}$ propeptide (h)	$t_{1/2}$ VWF (h)	Blood group
Normal	2.3	7.0	
C1130F	3.2	1.6	O
C1149R	2.6	1.5	A
C2671Y	6.0	1.3	O

Mutations at C1130, C1149 and C2671 leave ADAMTS13-dependent proteolysis unaffected
Rapid clearance could be due to increased susceptibility of VWF mutants to degradation by ADAMTS13. The susceptibility of the recombinant mutants for ADAMTS13 was therefore assessed in degradation assays. Representative results from the protease assay of rVWF/C1130F and rVWF/C2671Y are shown in Figure 3. No or little degradation was detected in the absence of urea. However, the presence of 1.0 M urea resulted in a gradual disappearance of the higher multimers (Figure 3). Due to impaired multimerization of rVWF/C1130F and rVWF/C1149R, the sensitivity for ADAMTS13 could only be interpreted for the respective cotransfections with wt-VWF, which are also more representative for the situation in the heterozygous patients (Figure 3). At all urea concentrations and at all incubation times tested, the VWF mutants were indistinguishable from wt-VWF and no evidence was found that the accelerated disappearance from the circulation is caused by an elevated susceptibility towards ADAMTS13-mediated cleavage.

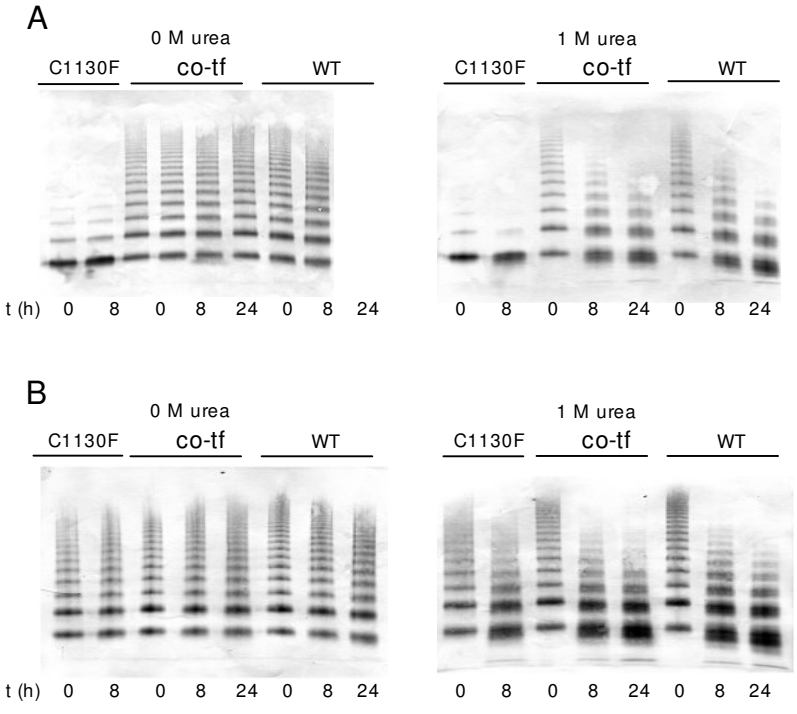

Figure. 3. ADAMTS13-mediated proteolysis of recombinant wild-type and mutant VWF
rVWF/C1130F (**panel A**), rVWF/C2671Y (**panel B**), wt-VWF (WT) alone or VWF obtained from co-transfections (Co-tf) were incubated with recombinant ADAMTS13 for 0, 8 or 24 hours (t) in the absence or presence of 0.1, 0.5, 1.0, 1.5 M urea. The results from 0 and 1.0 M urea are depicted. The degree of proteolysis was assayed on SDS-agarose gel electrophoresis under non-reducing conditions. The results for rVWF/C1130F are representative for rVWF/C1149R (data not shown).

Increased clearance of VWF mutants in VWF-deficient mice

The clearance of mutant VWF was studied in a murine model in which VWF clearance is independent of ADAMTS13-mediated proteolysis and independent of the extent of VWF multimerization[7]. VWF mutants produced by stable cell-lines were purified from conditioned medium. Multimer analysis revealed a normal multimeric pattern for rVWF/C2671Y. rVWF/C1130F and rVWF/C1149R consisted mainly of low molecular weight multimers, whereas co-expression of rVWF/C1149R resulted in the reappearance of higher multimers. These patterns are similar to that of the mutants produced by transiently transfected cells (Figure 3 and [12]). Purified proteins were injected intravenously in VWF-deficient mice and residual plasma-levels were measured. The mutants and wt-VWF displayed a similar recovery after injection (Table 3). In contrast, all four mutants were cleared from the circulation more rapidly than wt-VWF (Table 3, Figure 4). In the murine system, wt-VWF disappears from plasma in a biphasic manner, characterized by a rapid initial phase and a slow secondary phase [7]. As for wt-VWF, the mutants disappeared in a biphasic manner, but

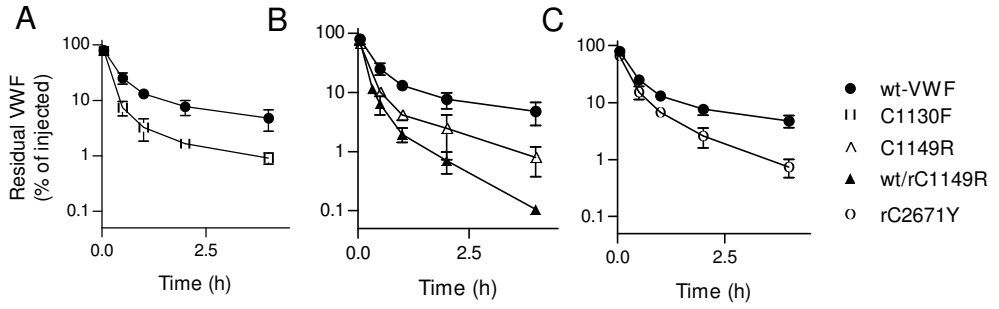

Figure 4. In vivo survival of recombinant VWF mutants
VWF-deficient mice were injected intravenously with purified wt-VWF, rVWF/C1130F (**Panel A**), rVWF/C1149R (Panel B), wt-VWF-rVWF/C1149R (**Panel B**) or rVWF/C2671Y (**Panel C**) at a concentration of 5 μg/mouse. Plotted is the residual VWF:Ag in plasma relative to the amount injected versus time after injection. For clarity, only data between 3 min and 4 h are shown. Data for wt-VWF were obtained in a previous study 7, and are shown for comparison. Pharmacokinetic parameters derived from the complete data set (from 3 min to 24 h) are summarized in Table 3. Data represent the mean±S.D. of 3-6 mice for each time-point.

Table 3. Pharmacokinetic parameters of the clearance of recombinant VWF in mice.

	Recovery (% of injected)	mean residence time (h)	$t_{1/2}\alpha$ (min)	$t_{1/2}\beta$ (h)
wt-VWF*	79±14	2.8±0.7	12.6±0.9	3.0±0.9
rVWF/D2509G*	82±5 (p>0.05)	2.3±0.5 (p>0.05)	12.0±2.2 (p>0.05)	2.2±0.2 (p>0.05)
rVWF/C1130F	72±6 (p>0.05)	0.7±0.2 (p=0.007)	6.0±1.4 (p=0.002)	1.1±0.5 (p=0.032)
rVWF/C1149R	67±10 (p>0.05)	0.8±0.4 (p=0.011)	7.5±2.5 (p=0.028)	1.1±0.5 (p=0.032)
wt-VWF-rVWF/C1149R	74.9±9 (p>0.05)	0.4±0.1 (p=0.0042)	5.5±0.4 (p=0.0002)	0.7±0.1 (p=0.012)
rVWF/R1205H*	97±8 (p>0.05)	0.3±0.1 (p=0.004)	7.6±0.2 (p=0.0007)	0.3±0.03 (p=0.007)
rVWF/C2671Y	63±8 (p>0.05)	0.7±0.1 (p=0.006)	8.9±1.5 (p=0.022)	0.7±0.2 (p=0.012)

*Data were obtained during a previous study [7]. wt-VWF is reference for comparisons. rVWF/D2509G and rVWF/R1205H are shown to illustrate the normal and decreased half-life of other recombinant mutants.

data analysis revealed that the initial rapid phase (t1/2α), the slow secondary phase (t1/2β) and mean residence time were significantly reduced compared to the values obtained for wt-VWF (Table 3). Thus, the mutations C1130F, C1149R and C2671Y per se are associated with accelerated clearance of VWF.

Table 4. Functional analysis of recombinant VWF mutants.

	GpIbα binding Half-maximal binding (nM)	Collagen binding Kd_{app} (nM)	FVIII binding Kd_{app} (nM)	Platelet adhesion Surface coverage (%)
wt-VWF	1.2±0.2	9.4±0.6	0.51±0.04	77.3±6.3
rVWF/C1130F	1.0±0.1	47.4±1.0	2.3±0.1	80.9±8.8
rVWF/C1149R	3.9±1.1	53.4±1.4	10.6±0.4	49.4±16.1
rVWF/C2671Y	1.0±0.2	8.3±0.6	0.33±0.02	79.2±9.8
Dimeric control*	n.d.	67.5±1.2	0.38±0.06	70.5±6.4

*Dimeric control was: rVWF-D'-A3 for collagen binding, rVWF-A1-CK for platelet-adhesion and rVWF/D'-D3 for FVIII binding. All dimeric controls have been described previously 7. n.d.: not determined

Functional characterization of recombinant mutants rVWF/C1130F, rVWF/C1149R and rVWF/C2671Y

Because the accelerated clearance of the mutant VWF is due to the mutations per se, we examined whether the mutants have functional or structural characteristics in common that point to a region in VWF responsible for the increased clearance. The results of a number of functional parameters (ie. FVIII-, GpIbα- and collagen-binding, platelet-adhesion under flow-conditions) are summarized in Table 4. Briefly, mutant rVWF/C2671Y was similar to wt-VWF in all assays tested. Mutant rVWF/C1130F displayed normal GpIbα-binding and platelet-adhesion capacity. Furthermore, FVIII-binding was reduced 5-fold for this mutant, while collagen-binding was reduced to a level similar to a dimeric control protein. Mutant rVWF/C1149R was most severely affected; apart from collagen-binding that was reduced to the level of the dimeric control, all other functions were considerably reduced compared to the control proteins.

In view of the multimerization-defects of rVWF/C1130F and rVWF/C1149R, we also tested the interaction with the propeptide. This interaction mediates intracellular multimerization and targeting of mature VWF to the storage-organelles and occurs under slightly acidic conditions. The interaction between VWF and propeptide was investigated at various pHs (Figure 5). For wt-VWF a pH-dependent increase in affinity was observed with lower affinity at higher pH (pH7.4, K_D,app=1.0±0.1 mM and pH5.2, K_D,app=0.08±0.02 mM). A similar pattern was found for rVWF/C2671Y. However, binding of propeptide was markedly reduced for mutants rVWF/C1130F and rVWF/C1149R (K_D,app>2 mM at all pHs tested). Again, rVWF/C1149R was less efficient compared to rVWF/C1130F. These data indicate that mutations within the VWF-D'D3 domain are not only associated with increased clearance, but also may lead to sub optimal interactions with ligands that bind to this particular region of the VWF molecule.

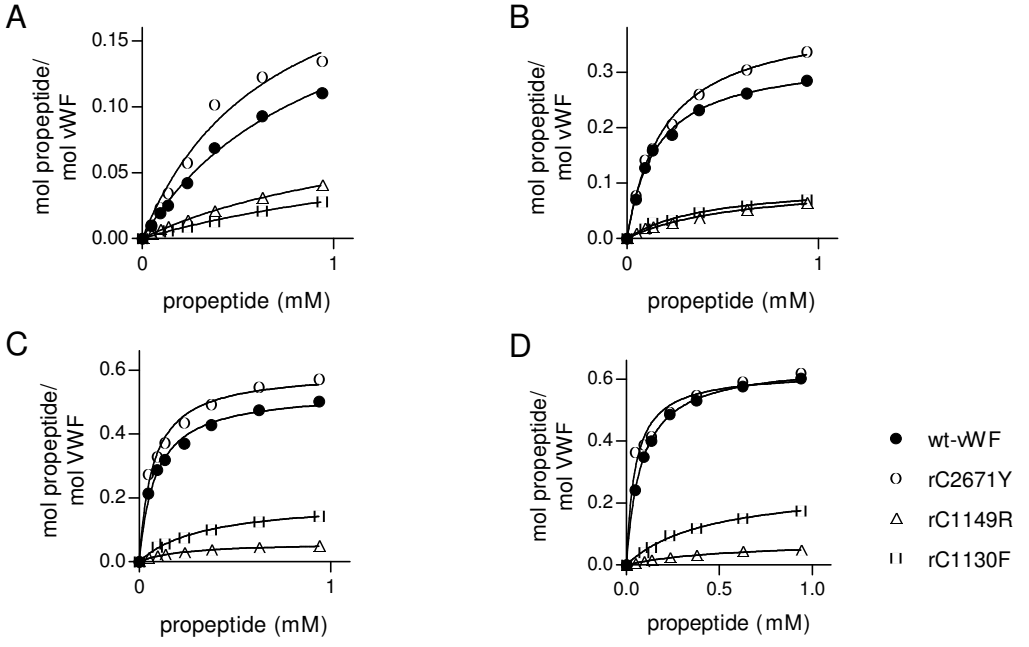

Figure 5. Binding of propeptide to immobilized VWF.
wt-VWF, rVWF/C1130F, rVWF/C1149R, and rVWF/C2671Y immobilized onto a CM5 sensor-chip (10-12 fmol/mm2) were incubated with various concentrations of purified propeptide (0-1.0 mM). Incubations were performed in 125 mM NaCl, 25 mM HEPES (pH 7.4 (**Panel A**) or pH 6.8 (**Panel B**)), 125 mM NaCl, 25 mM $(CH_3)_2AsO_2Na$ (pH 5.8 (**Panel C**) or pH 5.2 (**Panel D**)) at a flow rate of 10 ml/min for 4 min at 25°C. Response at equilibrium (indicated as mol propeptide/mol VWF) are plotted against concentration of propeptide. Please note the different scales of the Y-axes. Data represent the mean of duplicate experiments (range less than 10 %).

Discussion

Circulating levels of VWF need strict regulation, as levels that are too low are associated with an increased bleeding tendency [1], whereas levels that are too high predispose to an increased risk of cardiovascular mortality [24]. Low levels of VWF can result from decreased synthesis, impaired secretion, increased clearance or combinations thereof. Several mutations lead to impaired synthesis or secretion of VWF, however, little is known about the relationship between amino acid variations in VWF and its survival in the circulation. In the present study we evaluated the effect of three VWD-associated mutations on the survival of VWF. These mutations were selected on basis of discrepancies between VWF levels measured in in vitro expression experiments and the relatively lower actual antigen values in the patients' plasmas [8,10,12]. Such discrepancies may indicate the presence of mechanisms other then synthesis and secretion that contribute to the low VWF levels.

We have obtained several lines of evidence of increased clearance of mutants VWF/C1130F, VWF/C1149R and VWF/C2671Y. First we analyzed in the patients the propeptide/VWF:Ag ratio, which is the resultant of a dissimilar survival of these proteins in the circulation [14,23]. The VWF:Ag levels were much more reduced than propeptide levels, resulting in ratios that were increased up to 3-fold compared to normal individuals or unaffected family members (Figure 1). Second, VWF disappeared from the circulation 4-5 fold more rapidly in the patients upon DDAVP-treatment (Figure 2B). Third, we performed detailed analysis of in vivo clearance of recombinant VWF in a model employing VWF-deficient mice [7]. Both the initial and secondary phase of the clearance of rVWF/C1130F, rVWF/C1149R and rVWF/C2671Y were accelerated (Figure 4), which was reflected by a mean residence time that was reduced 4-fold (Table 3). Thus, the mutations C1130F, C1149R and C2671Y are each associated with a reduced survival of VWF in the circulation. Because also the heterozygous wt-VWF-rVWF/C1149R mimicked the increased clearance of the protein in the patients, it seems that at least some of these mutations may have a predominant effect on the clearance of the mutated protein. Furthermore, we have recently shown that also mutation R1205H results in increased clearance of VWF [7]. A preliminary report has recently described a mutation S2179F in the VWF-D4 domain, which seems to have a similar effect on VWF clearance [25]. This indicates that several mutations in VWF predispose to increased clearance suggesting it to be a more general phenomenon than previously anticipated. Indeed, a decreased survival of VWF upon DDAVP treatment in a cohort of VWD-type 1 patients has recently been reported [26].

This conclusion is of importance with respect to treatment of VWD patients. The goal of treatment is to correct the deficiency of VWF and FVIII, either by transfusion with plasma-derived FVIII/VWF concentrates or DDAVP administration. The latter leads to the release of endogenous VWF from storage organelles, which is associated with a 3 to 5-fold increase of VWF levels. However, if due to a mutation the survival of endogenous VWF is reduced, the initial response to DDAVP may be normal, but the effect is only short-lasting because the endogenous VWF is cleared rapidly. In some of these patients it may be more appropriate to treat with FVIII/VWF concentrates. In clinical practice the initial response to DDAVP is used to judge the effectiveness of desmopressin, but this may be insufficient for several patients as it does not take clearance into account. Therefore we suggest that, after a DDAVP test-infusion, VWF:Ag is monitored over a sufficient length of time and we propose the implementation of the propeptide/VWF:Ag ratio in the diagnosis of VWD.

The question remains why the various mutations are associated with increased clearance of the VWF protein. It has recently been shown that the presence of a Tyr to Cys polymorphism at position 1584 results in increased susceptibility to the VWF-cleaving protease ADAMTS13 [27]. Since the patient harboring the C2671Y mutation displays relatively high plasma levels of VWF degradation products [28], we tested whether increased clearance could result from an increased susceptibility to the ADAMTS13-protease. However, we did not find evidence of increased proteolysis (Figure 3). Moreover, normal proteolysis was also reported for VWF/R1205H [29], another mutant that displays increased clearance [7]. Final evidence that the increased clearance of the various mutants is independent of ADAMTS13

was provided by the rapid clearance in the mouse model, as murine ADAMTS13 does not recognize human VWF. It should be noted that our study has not examined the potential role of thrombospondin-1, a plasma depolymerase that is able to modulate VWF multimer size [30].

Increased clearance may also be due to disturbed structural integrity of the mutant proteins. We have tried to identify whether the mutant proteins have functional or structural characteristics in common that may be associated with the phenomenon of increased clearance. Several parameters were examined, but none of these functional tests pointed to a particular region within the VWF molecule that seems to be consistently associated with abnormal clearance. Mutant rVWF/C2671Y was normal for all functions, indicating that this mutation results in local changes within the molecule only. This could suggest that this part of VWF contributes to interactions with clearance receptors. All other mutations that we tested so far, i.e. rVWF/C1130F, rVWF/C1149R and rVWF/R1205H, are located within a relatively short stretch in the D3 domain. However, the impact of these mutations on VWF function differs considerably. Whereas mutant rVWF/R1205H has normal interaction with FVIII [7], both rVWF/C1130F and rVWF/C1149R display impaired FVIII binding (Table 4). Mutations C1130F and C1149R further resulted in a lack of proper multimerization (Figure 3 [12]), which was not seen with rVWF/R1205H [7]. A combined effect on FVIII binding and multimerization has been described for several other mutations, and seems to be related to a local distortion of the secondary structure [31]. Incomplete multimerization is a consequence of a sub optimal interaction with the propeptide, which is involved in intracellular multimerization of VWF [32,33]. Indeed, mutants rVWF/C1130F and rVWF/C1149R displayed defective binding of propeptide (Figure 5). Thus, the lack of multimerization observed for these mutants can readily be explained by an almost complete absence of propeptide binding.

In conclusion, our study shows that the phenomenon of increased clearance in VWD occurs more often than previously anticipated. This may have major implications for the therapeutic strategies that rely on the temporary rise of endogenous VWF after DDAVP administration. New therapeutic approaches based on the inhibition of VWF clearance could be developed as an adjuvant to optimize treatment of these patients. However, more insights into the molecular mechanisms mediating VWF clearance are needed in this regard.

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CHAPTER 4

CHAPTER 4

Prediction of Factor VIII half-life in severe Haemophiliacs: distinct approaches for blood group O and non-O patients

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Abstract

It has been well-established that the in vivo survival of factor VIII (FVIII) is dependent on the presence of its carrier-protein von Willebrand factor (VWF). Moreover, it has been reported that the half-life of FVIII correlates to VWF antigen pre-infusion levels. Here, we explored the possibility that VWF pre-infusion levels can be used to predict FVIII half-life values in order to avoid individual half-life studies in haemophilia A patients. Our analysis showed that VWF antigen levels correlated well with FVIII half-life in blood-group non-O, but poorly in blood-group O patients. This poor correlation for blood-group O patients could be considerably improved when VWF/propeptide ratio was used. We performed multivariate regression analysis using VWF and propeptide as open parameters. This resulted in accurate fitting of individual data-points, allowing us to predict FVIII half-life values using preinfusion VWF and propeptide values with an accuracy of 2 h compared to the actual FVIII half-life value. We feel that our analysis will be beneficial in predicting FVIII half-life values in haemophilic patients and can be used for optimizing prophylactic regimens.

Introduction

The severe bleeding disorder haemophilia A is caused by defects in the gene encoding coagulation factor VIII and affects 1 in approximately 5000 males [1]. In plasma, FVIII circulates in a tight non-covalent complex with von Willebrand factor (VWF). The formation of this complex is of physiological importance to maintain appropriate plasma levels of FVIII [2,3]. Indeed, patients lacking VWF (von Willebrand disease (VWD) type 3) have not only a secondary deficiency of FVIII, but also a strongly reduced half-life of intravenously administered FVIII [4].

Current treatment of haemophilia A mainly involves replacement therapy using purified plasma-derived or recombinant FVIII. Since the early 70's, treatment protocols started to shift from on-demand towards prophylactic treatment. Initially, this was applied for patients who had already experienced several joint bleeds [5]. More recently, prophylactic treatment has become more widespread and is often started after 1 or 2 joint bleeds. Although prophylactic treatment has been proven to be beneficial for the patient, it requires regular infusions (up to 3 times per week) because of the relative short half-life of FVIII. The average half-life of FVIII in haemophilia A patients is 12 hours, but a large variation between individuals (6-29 h) has been observed [6,7]. Due to this large variation in half-life, it requires individual half-life studies in order to optimize treatment-protocols [8].

Few factors have been identified that are associated with this variation in half-life, the major one being pre-infusion VWF levels: a correlation between pre-infusion VWF levels and FVIII half-life has been described in at least two separate studies [6,9]. Since VWF levels are on average 25 % lower in blood group O individuals compared to blood group non-O individuals (for review see [10]), it is not surprising that FVIII half-life is found to be shorter in haemophiliacs with blood-group O than in non-O patients [9].

Recently, we have described a cohort of 38 severe haemophilia A patients, in whom FVIII half-life has been determined [7]. We have re-analyzed these data in order to investigate whether pre-infusion VWF data may be used to reliably predict FVIII half-life in these patients. Our analysis employing regression-based data-fitting revealed that pre-infusion VWF are a useful tool in predicting FVIII half-life values, providing that VWF-propeptide levels are taken into account. In addition, different behavior of data obtained from blood-group O and non-O patients was observed.

Table 1: Deviation of parameters from fitted line.

	VWF:Ag	VWF/propeptide	Regression model
		Blood-group non-O	
Deviation mean ± SD (%)	15.8 ± 13.5	66.7 ± 62.0	12.5 ± 8.6
Range (min-max)	-21.8 – 61.7	-146.4 – 231.6	-20.8 –34.7
		Blood-group O	
Deviation mean ± SD (%)	38.6 ± 33.1	20.6 ± 14.1	12.9 ± 9.4
Range (min-max)	-130.1 – 78.6	-48.4 – 46.8	-26.6 – 35.3

For each set of data points (Figure 1), deviation from the fitted line and other relevant parameters are presented in the table.

Patients, Materials and Methods

Patients

Thirty-eight patients with severe haemophilia A (<1 % residual FVIII activity) that were included in the present analysis have been described in detail elsewhere [7,11]. None of the patients were HIV-positive, had liver failure, fever in the previous 48 h, low platelets, increased prothrombin time, low factor V, signs of liver cirrhosis on ultrasound, severe bleeding or surgery in the previous 3 months, or antibodies against FVIII (as assessed by an immunosorbent-assay or a Bethesda-assay (sensitivity <0.3 Bethesda Units/ml)). Nineteen patients were blood-group O, and nineteen were non-O (12 A, 5 B, 2 AB). Eligible patients gave informed consent. The Medical Ethics Committee of our institute gave approval to the study.

Study design

The results of the FVIII half-life determination have been reported elsewhere [7].

Laboratory assays

VWF antigen and propeptide antigen were determined and values were converted into molar concentrations as described [12,13,14]. Antigen levels were determined blindly, being unaware of corresponding FVIII half-life values.

Data analysis

VWF antigen or VWF/propeptide ratios were plotted against FVIII half-life values that were determined previously [7]. Best fit of these plots were calculated using linear regression analysis (GraphPad Prism version 4.0 for Windows, GraphPad Software, San Diego, CA). Multiple variable regression analysis was performed using SPSS software (SPSS Inc., Chicago, IL). Relative deviation of individual data points from the fitted line were calculated, the statistical analysis of which is summarized in table 1.

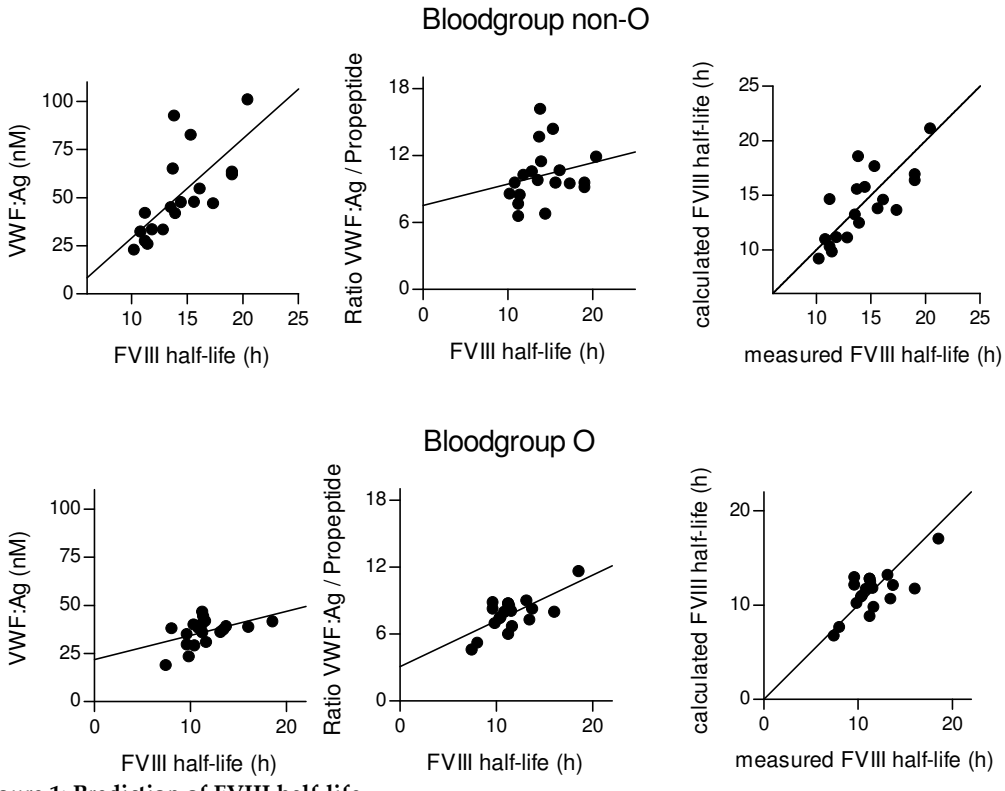

Figure 1: Prediction of FVIII half-life

Panel A-C show analysis of blood group non-O patients and **panel D-F** for blood group O patients. VWF antigen levels (Panel A and D) and VWF:Ag/propeptide ratio's (Panel B and E) were plotted against FVIII half-life. In panel C and F calculated FVIII half-life (derived from the regression-model) is plotted against measured FVIII half-life.

Results and discussion

In a previous study, we have determined the FVIII half-life of 38 severe haemophilia A patients [7]. FVIII half-life varied between 7.4 and 20.4 h. In concordance with a report by Vlot *et al*.[9], the average FVIII half-life was shorter in blood-group O patients than in non-O patients (11.5±2.6 h versus 14.3±3.0 h; p=.0044). Furthermore, average VWF levels were lower in blood-group O patients than in non-O patients (36.2±7.0 nM versus 51.1±22.2 nM; p=.00854). In contrast, propeptide levels were similar for both groups (4.7±1.0 nM versus 4.9±1.6 nM; p=.61). As a consequence, propeptide/VWF ratios were significantly increased in blood-group O patients compared to non-O patients (0.13± 0.03 versus 0.10±0.02; p=.0011; Figure 1B and 1E). Although formal evidence still needs to be provided, these differences in propeptide/VWF ratios between blood groups strongly point to VWF being cleared more rapidly in blood-group O persons than in those with blood-group non-O [15,16].

Given the blood-group dependent differences, both O and non-O patients were analysed separately with regard to the correlation between VWF and FVIII half-life. As shown in Figure 1A, a fair correlation between VWF antigen levels and FVIII half-life was observed in blood-group non-O patients (pearson-rank=0.70; p=.001). However, this correlation appeared to be less obvious in O-patients (pearson-rank=0.47; p=.044; Figure 1D). We considered the possibility that the correlation between VWF antigen and FVIII half-life could be influenced using VWF/propeptide ratio in stead of VWF antigen. Surprisingly, this approach worked adversely for blood-group non-O patients, as it resulted in a non-significant correlation with FVIII half-life (pearson-rank=0.23; p=.34; Figure 1B). In contrast, the correlation was markedly improved by this approach for blood-group O patients (pearson-rank=.70; p=.001; Figure 1E). Apparently, blood-group status of haemophilic patients should be considered when considering VWF as a predictive marker for FVIII half-life.

Despite the reasonable pearson-rank coefficient values of 0.70 for both blood-group O and non-O patients, it was less satisfying that we could observe considerable deviation of individual values from the fitted line. This deviation varied between -21.8 % and +61.7 % for non-O patients, while values deviated between -48.4 % and + 46.% for the O patients (Table 1). We therefore decided to investigate whether fits could be optimized, via multivariable regression analysis using VWF antigen and propeptide levels as open parameters. This approach resulted in correlations that were characterized by a more accurate fit for both blood-group non-O and O patients (Figure 1C and 1F, respectively). Indeed, deviation of values was reduced, in that they deviated between -20.8 % and +34.7 % and between -26.6 % and + 35.3 % for O and non-O patient groups, respectively (Table 1). The best fitted lines could be described employing the following formulae:

For blood-group non-O patients: $1.74 \times [\text{propeptide}] + 0.54 \times [\text{VWF}] / [\text{propeptide}]$

For blood-group O patients: $1.47 \times [\text{VWF}] / [\text{propeptide}]$

In both cases, correlation was highly significant (pearson-rank=0.76; p=.0002 for blood-group O patients; Figure 1C; and pearson-rank=0.70; p=.001 for blood-group O patients; Figure 1F). In absolute terms, the average deviation of individual data points was 1.8±1.2 h (95% CI 1.2-2.3 h) and 1.5±1.1 h (95 % CI 1.0-2.0 h) for blood-group non-O and O patients.

In conclusion, our approach allows the prediction of FVIII half-life within a range of about 2 h using VWF and propeptide levels. We feel that our analysis will be beneficial in predicting FVIII half-life values in haemophilic patients. In addition, our data strongly support the view that clearance of FVIII is dictated by VWF. Since clearance of VWF itself differs between individuals of different blood-groups, it is of importance to take these differences into account when considering clearance of FVIII.

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CHAPTER 5

CHAPTER 5

Macrophages in the liver and spleen contribute to the clearance of Factor VIII, von Willebrand factor and its complex

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Abstract

Von Willebrand Factor (VWF) and factor VIII (FVIII) circulate in complex in circulation. Complex formation stabilizes FVIII and is essential to promote survival of FVIII in the circulation. Important is that the half-life of FVIII mimics the half-life of the VWF/FVIII complex, suggesting that FVIII is predominantly removed from the circulation via VWF rather then as a separate entity. The aim of the present study was to define the molecular strategy by which VWF is cleared from the circulation. We show here that in particular liver and spleen are efficient in the uptake of VWF. Analysis of liver and spleen sections of VWF-deficient mice infused with FVII, VWF or the VWF/FVIII complex revealed co-localisation of all proteins with macrophages in both tissues. We also tested the contribution of these cells to the in vivo clearance of VWF. Mice were treated with gadolinium chloride ($GdCl_3$) prior to VWF infusion, to decrease macrophage cell numbers and function. This resulted in a 2-fold increase (0.7±0.1 vs. 1.3±0.4 U/ml; $p<0.05$) of endogenous VWF in wild-type mice pre-treated with $GdCl_3$. In addition, the survival of infused VWF was prolonged almost 2-fold (MRT= 2.7 h vs. 4.5 h) in VWF-deficient mice. To study the uptake of the VWF by macrophages in more detail, we developed an in vitro model using macrophages. We showed binding of VWF and FVIII to human macrophages. Moreover, radiolabelled VWF bound to these cells in a dose-dependent and saturable manner (half-maximal binding at 13.5 µg/ml). Specificity was shown by a dose-dependent decrease in binding of labelled VWF when increasing amounts of non-labelled VWF were added. Association of radiolabelled VWF to macrophages was followed by a rapid uptake and subsequent degradation of the internalised protein. Binding and uptake was also visualized using a VWF-green fluorescent protein (VWF-GFP) fusion protein, confirming the capacity of macrophages as cells that mediate the clearance of VWF.

Introduction

Von Willebrand Factor (VWF) is a multimeric plasma protein that is produced in endothelial cells and megakaryocytes. VWF is synthezised as a pre-pro peptide and undergoes several post-translational modifications, including glycosylation, multimerization and proteolytic cleavage of its propeptide. This results in large VWF multimers that are either secreted in plasma or stored in storage organelles. During haemostasis VWF facilitates platelet binding to the damaged vessel wall, acting as a molecular bridge between the subendothelial matrix and platelet receptor GPIbα [1,2]. Part of the VWF molecules circulate in complex with coagulation factor VIII (FVIII). This interaction stabilizes FVIII and prevents its premature clearance from the circulation [3,4]. FVIII acts as a cofactor for activated factor IX (FIX) in the coagulation cascade and the physiological importance of FVIII is illustrated by the severe bleeding tendency (Haemophilia A) associated with the functional absence of the protein [4]. FVIII is synthesized in various tissues, including liver, spleen and kidney [5,6]. A number of potential clearance receptors for FVIII have been identified, including members of the low-density lipoprotein (LDL) receptor family, heparan-sulfate proteoglycans and asialoglycoprotein-receptor (for review see [7,8]). It should be noted that binding of FVIII to these receptors is inhibited by VWF, suggesting that only free FVIII (about 2-5 % of total FVIII) is available for clearance by these receptors. Therefore, plasma levels of FVIII depend strongly on VWF plasma levels, since complex formation with VWF increases FVIII half-life [7,9].

VWF plasma levels in normal individuals show a wide variation that can be influenced by several factors. Blood-group dependent modifications of the glycosylation profile may affect VWF antigen levels, since individuals with blood group O display 25% lower VWF levels when compared to non-O carriers [10]. Also other carbohydrate structures may affect the clearance rate of VWF, such as O-linked carbohydrates and terminal sialyl-groups [11,12].

Steady state plasma levels of VWF need to be tightly regulated in order to optimally serve the haemostatic process [1]. Patients suffering from Von Willebrand Disease (VWD) type 1 show decreased VWF levels and in some cases an increased propeptide/VWF ratio, which is believed to be a surrogate marker for VWF clearance [13,14]. This indicates that a modified VWF turnover can contribute to the pathogenesis of VWD.

Relatively little is known concerning molecular mechanisms responsible for removal of VWF from the circulation. Injection of human plasma-derived (pd-) VWF in VWF-deficient mice revealed that VWF is mainly targeted to the liver, while small amounts of VWF are digested by other organs such as spleen and kidneys [15]. However, the cellular destination of VWF in these organs is unclear. Moreover, no information exists as to whether VWF and FVIII are cleared by these cells as a singular entity or whether different cells are involved in the clearance of FVIII, VWF or the VWF/FVIII complex. The aim of the present study was to identify cells contributing to the clearance of these proteins. Our data indicate that macrophages play an important role in the removal of FVIII and VWF from the circulation.

Materials and Methods

Proteins and antibodies

Plasma-derived (pd)-VWF was purified from Haemate P (Behring, Marburg, Germany) as described [16]. A GFP-tagged VWF construct (VWF-GFP) was kindly provided by Dr. S Meijer, Sanquin Research, the Netherlands [17]. Recombinant FVIII was derived from Refacto (Wyeth, Madison, NJ). Polyclonal rabbit antibodies against human VWF were obtained from DakoCytomation (Glostrup, Denmark) and sheep polyclonal anti-human von Willebrand factor immunoglobulins were purchased from Biozol (Eching, Germany). Monoclonal rat anti-mouse CD68 and rat anti-mouse F4/80 were obtained from AbD Serotec (Oxford, UK), whereas monoclonal mouse anti-human CD16 antibodies were purchased from BD Pharmingen (San Diego, CA). Monoclonal mouse anti-human FVIII antibodies (CLB-CAgA) were kindly provided by Dr. J. van Mourik, Sanquin Research, the Netherlands and monoclonal human anti-human FVIII antibody (LE2E9) were a gift from Dr. J.M. Saint-Remy, KU Leuven, Belgium [18]. Polyclonal horseradish peroxidase (HRP)-conjugated antibodies were purchased from DakoCytomation (Glostrup, Denmark). Rhodamine conjugated donkey anti-sheep immunoglobulins were obtained from Santa Cruz (CA, USA). Other rhodamine (TRITC)-conjugated and fluorescein (FITC)-conjugated secondary antibodies were purchased from Jackson immunoresearch laboratories (West Grove, PA).

Mice and tissue collection

The VWF-deficient mice [19] and wild type mice used in the present study were on a C57BL/6J background and were used between 8 an 12 weeks old. Housing and experiments were done as recommended by French regulations and the experimental guidelines of the European Community. VWF-deficient mice were injected with human recombinant VWF (10 μg/mouse), human recombinant FVIII (0.5 μg/mouse) or both FVIII and VWF (0.5 μg and 10 μg per mouse, respectively), which were pre-incubated for 30 min prior to injection in order to allow complex formation. After injection (5 min for FVIII and 30 min for VWF or VWF/FVIII) mice were bled, sacrificed and tissue was collected, perfused with PBS, embedded in Tissue-Tek OGT compound (Miles Laboratories, Elkhart, IN) and immediately frozen in liquid nitrogen. Tissue was cut into 8 mm sections on a freezing microtome and mounted on slides.

Chemical treatment of mice

Gadolinium chloride ($GdCl_3$, 50mg/kg) was given to mice via intravenous tail injection 24 h prior to VWF injection. Control mice received equivalent injections of saline solution. Blood was collected and processed for VWF antigen analysis. Tissues were collected as described. Depletion of macrophages in the liver, caused by the $GdCl_3$ was quantified as the average number of Kupffer cells per field from at least 10 randomly selected fields per section. Kupffer cells were identified as CD68 positive cells with an appropriate nuclear morphology and sinusoidal location.

Clearance of purified recombinant VWF in mice

Clearance of recombinant wt-VWF and mutant VWF has been analysed as described [15,19]. Three to six mice were used for each time-point, and each mouse was bled only once. VWF antigen levels were quantified in mouse plasma as described before [20]. Normal pooled plasma was used as a reference.

Immunohistochemistry

Cryostat sections were immunostained by the indirect immunoperoxidase method using the following antibodies: monoclonal rat anti-mouse CD68, monoclonal rat anti-mouse F4/80 or peroxidase-conjugated polyclonal rabbit anti-human VWF. As second step, sections were incubated with peroxidase-conjugated goat anti-rat immunoglobulin or goat anti-rabbit immunoglobulin. The peroxidase labelling was visualized using DAB (Vector Laboratories, Burlingame, CA) as substrate, resulting in a brown staining. Slides were counterstained with hematoxylin and immunohistochemically stained sections were examined by light microscopy

Immunofluorescence

Cryostat sections were immunostained using the following antibodies. For detection of macrophages in mouse tissue monoclonal rat anti-mouse CD68 or monoclonal rat anti-mouse F4/80 were used. TRITC-conjugated goat anti-rat immunoglobulins were used as secondary antibodies. For the detection of VWF we used either polyclonal rabbit anti-human VWF (with FITC-conjugated goat anti-rabbit antibodies as secondary antibodies) or polyclonal sheep anti-human VWF (with TRITC-conjugated donkey anti-sheep antibodies as secondary antibodies). For the detection of FVIII we incubated sections with LE2E9, followed by incubation with FITC-conjugated anti-human antibodies. Afterwards the FITC signal was amplified using alexa fluor 488 signal amplification kit (Invitrogen, Maryland, USA). All antibodies were incubated for 30 min at room temperature. After the staining procedure, sections were embedded in Mowiol mounting medium containing 2.5% DABCO (Fluka Chemie AG, Buchs, Switzerland) and analyzed using a Leitz DMIB fluorescence microsope, with Planapo objective (Leica, Voorburg, the Netherlands), interfaced with a Leica TCS4D confocal laser microscope (Leica Lasertechnik, Heidelberg, Germany).

Radiolabeling of pd-VWF

Pd-VWF was labelled with $Na^{125}I$ (Amersham Biosciences Inc, UK) using the IODO-GEN method (Pierce Chemical. Co, Rockford, IL). Radiolabeled protein was separated from free iodine on a PD10-disposable column and equilibrated in Hepes-buffered saline/0.005% Tween-20. The content of free iodine was routinely less than 6% as determined by precipitation in 10% trichloroacetic acid.

Preparation of human macrophages

Monocytes were isolated from peripheral blood, drawn from healthy individuals, by centrifugation on a Ficoll-Paque gradient (GE Healthcare Bio-sciences Corp., N.J., U.S.A) and magnetically sorted using a CD14+ magnetic bead isolation kit (MACS, Miltenyi Biotec Inc., CA, USA). Cells were then seeded on glass coverslips coated with coating buffer (phosphate-buffered saline (PBS) containing 4% bovine albumin, BSA) in 24-well culture plates in growth medium (RPMI-1640 medium supplemented with 5% heat-inactivated fetal calf serum, 100 U/ml penicillin, 100 mg/ml streptomycin, 50 mM b-mercapto-ethanol). Cells were allowed to differentiate into macrophages for 5 days. Monocytic human THP-1 cells (ATCC TIB-202) were maintained in growth medium and differentiated into macrophages by growing the cells on glass coverslips, coated with coating buffer, in 24-well culture plates in growth medium supplemented with 20 ng/ml phorbol-12-myristate-13-acetate (Biomedicals Inc., Aurora, OH) for 72 h [21].

Cellular binding experiments

Macrophages were washed once with growth medium and incubated for 30 min at 37°C. Cells were washed once more with ice-cold RPMI-1640 medium and cooled for 15 min on ice. Subsequently either FVIII , pd-VWF, radiolabelled pd-VWF or VWF-GFP was added and incubated for 1 h while temperature was maintained at 4°C to avoid internalisation. Excess of unbound protein was removed by one subsequent wash with ice-cold RPMI-1640 medium, and coverslips were transferred to fresh wells containing ice-cold RPMI-1640 medium. Wells were washed once more and cells were either lysed with PBS/1% Triton-X100 for 1 h at room temperature or fixed with 4% paraformaldehyde for 15 minutes at 4°C. For experiments employing radiolabelled VWF, cells were lysed with PBS/1% Triton-X100. After lysis supernatant was collected and the amount of radioactivity was measured. In all experiments, controls were included to determine the amount of non-specific binding in the absence of cells. For all other experiments, cells were fixed, washed with PBS and presence of FVIII, VWF or CD16 was detected using the indicated antibodies. For staining of FVIII, FITC labeled CLB-CAgA was used (FITC labeling kit, Pierce Chemical. Co, Rockford, IL). VWF was detected using polyclonal rabbit anti-human VWF, followed by FITC-conjugated goat anti-rabbit antibodies, whereas CD16 was visualized using monoclonal mouse anti-human CD16 and TRITC-conjugated goat anti mouse immunoglobulins. Finally, coverslips were embedded in mowiol mounting medium containing 2.5% DABCO and analyzed as described under immunofluorescence. All treatments were performed in triplicate.

Cellular degradation experiments

Differentiated THP-1 macrophages were grown as described above in 24-well culture plates. Either radiolabeled pd-VWF (13.5 μg/ml) or VWF-GFP (50 μg/ml) was bound to the cells as described above. After binding for 1 h at 4°C, cells were washed with ice-cold RPMI-1640 medium to remove unbound protein and once with RPMI-1640 medium at 37°C. Cells were placed at 37°C to initiate degradation and incubation was continued at 37°C in a volume of 500 μl. When radiolabeled VWF was used, samples were taken at indicated time-points to determine the amount of degraded material. Degraded material is defined as the radioactivity that is soluble in 10% trichloroacetic acid. Conversion of cpm values into molar amounts of pd-VWF was performed. In all experiments, controls were included to determine the amount of nonspecific degradation in the absence of cells. When VWF-GFP was used, coverslips were transferred at the indicated time-points to wells containing ice-cold RPMI-1640 medium, to avoid further internalization and degradation. Wells were washed with ice-cold RPMI-1640 medium to remove the excess of unbound protein. Subsequently cells were fixed using 4% paraformaldehyde for 15 minutes at room temperature. Coverslips were embedded and analyzed as described above. All treatments were performed in triplicate and data presented here form a typical example of the mean.

Data analysis and Statistics

Analyses of data were performed using GraphPad Prism program (Graphpad Prism version 4.0 for Windows, GraphPad Software, San Diego, CA). P values below 0.05 were considered significant.

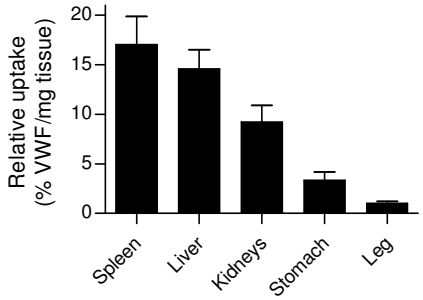


Figure 1 Biodistribution of radiolabeled pd-VWF
Radiolabeled pd-VWF was injected intravenously into VWF-deficient mice. After 27 hours mice were sacrificed and organs were collected. Indicated organs were weight and residual radioactivity was analyzed. The graph presents the percentage of residual radioactivity relative to the amount of injected protein per mg tissue. Data represent mean values ± SEM of three mice.

Results

Spleen and liver are most efficient in the uptake of VWF

Previously, we have demonstrated that the majority of VWF injected in VWF-deficient mice is targeted to the liver [15]. Since liver is the largest of the organs that were examined, we compared the efficiency by which liver takes up VWF to that of other organs. By calculating the uptake of VWF per weight of tissue, we found that apart from liver also spleen was particularly efficient in the uptake of VWF (14.6±3.4 and 17.0±4.9 % VWF/mg tissue, respectively; Figure 1). These amounts were higher when compared to other tissues, such as kidneys and stomach. Apparently, liver and spleen harbour cells that efficiently remove VWF from the circulation.

Macrophages as cellular destination for VWF in spleen and liver

In order to clarify the cellular destination of VWF in liver and spleen, tissues were isolated from VWF-deficient mice, injected with recombinant VWF (10 μg/mouse). Sections were stained for VWF, as shown in Figure 2. VWF was abundantly present in distinct regions within the spleen (Figure 2A). These regions seem to be located within the red pulp area, which is particularly rich in macrophages (Figure 2B). Co-staining for VWF and macrophages (using the anti-mouse macrophage marker F4/80 [22]) revealed considerable overlap (Figure 2C), indicating that VWF is co-localized with these macrophages. As for the liver sections, a pattern was observed upon VWF staining (Figure 2D) that displayed similarities to the pattern found upon staining for liver macrophages, ie. Kupffer cells using anti-murine CD68 antibodies (Figure 2E) [23]. Indeed, co-staining experiments for VWF and Kupffer cells demonstrated that the majority of VWF co-localizes with these macrophages (Figure 2F). These data indicate that macrophages contribute to the cellular uptake of VWF in both liver and spleen.

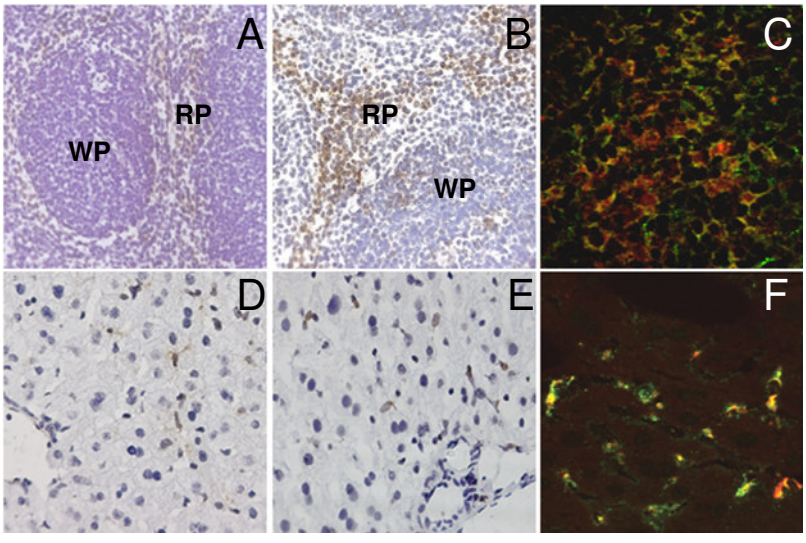


Figure 2 Identification of macrophages as main target for VWF in spleen and liver
Photomicrographs showing localization of pd-VWF and macrophages in spleen and liver after injection in VWF-deficient mice. **Panel A and B**; Spleen sections of VWF deficient mice treated with pd-VWF. Sections are counterstained using haematotoxylin (magnification 20x) RP: red pulp; WP: white pulp. **Panel A**; Section stained to detect VWF. Pd-VWF is localized in the RP of the spleen. **Panel B**; Section stained with the anti-mouse macrophage marker F4/80, showing the localization of macrophages in the RP. **Panel C**; Merged image; anti-mouse macrophage marker F4/80 (red), polyclonal anti-human VWF (green). Pd-VWF co-localizes with F4/80 positive macrophages in the RP. Original magnification,40x. **Panel D and E**; Liver sections of VWF deficient mice treated with pd-VWF (magnification 20x). Sections stained to detect CD68 positive Kupffer cells (**Panel D**) and human pd-VWF (**Panel E**). Panel F; Merged image; anti-mouse macrophage CD68 (red), anti-human VWF (green). Pd-VWF co-localizes with the CD68 positive Kupffer cells. Original magnification,40x.

Macrophages as cellular destination for FVIII in liver and spleen

To investigate whether cellular targeting of FVIII mimics that of its carrier-protein VWF, VWF-deficient mice were injected with recombinant human FVIII (0.5 μg/mouse). Analysis of sections stained using the monoclonal human anti-human FVIII antibody LE2E9 and anti-mouse macrophage specific antibodies (anti-F4/80 and anti-CD68 for spleen and liver, respectively), revealed that the majority of FVIII is targeted to cells positive for these macrophage markers (Figures 3A and B). Given the similarities in the cellular destination of FVIII and VWF in VWF-deficient mice, it was of interest to investigate whether injection of VWF and FVIII as a complex would result in a similar cellular distribution. Therefore, VWF-deficient mice were injected with recombinant FVIII and VWF (0.5 μg and 10 μg per mouse, respectively), which were pre-incubated for 30 min prior to injection in order to allow complex formation. Sections of liver and spleen were analyzed for the presence of FVIII and VWF. As shown in Figures 3C and D, more cells appeared positive for VWF alone than for FVIII or the VWF/FVIII complex. This probably originates from the approximately 20-fold molar excess of VWF that was injected. As for FVIII, the majority of FVIII staining overlaps staining for VWF (Figures 3C and D), indicating that both components of the FVIII and VWF

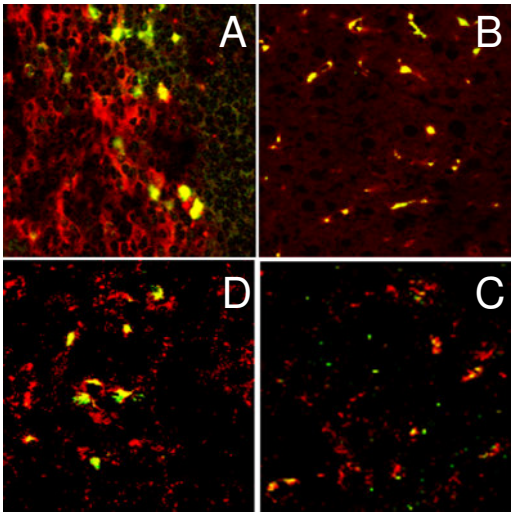


Figure 3 Localisation of FVIII and VWF/FVIII complex in spleen and liver
Photomicrographs showing localization of FVIII, VWF/FVIII complexes and macrophages in spleen and liver after injection in VWF-deficient mice. **Panel A**; Merged image of spleen section of VWF deficient mice treated with FVIII; anti-mouse macrophage marker F4/80 (red), monoclonal anti-human FVIII (green). FVIII co-localizes with F4/80 positive macrophages. **Panel B**; Merged image of liver section of VWF deficient mice treated with FVIII; anti-mouse macrophage marker CD68 (red), monoclonal anti-human FVIII (green). FVIII co-localizes with CD68 positive macrophages. Merged image of spleen (**Panel C**) and liver (**Panel D**) section of VWF deficient mice treated with VWF/FVIII; anti-human VWF (red), monoclonal anti-human FVIII (green).

complex are targeted to similar cells. It seems likely therefore that the VWF/FVIII complex is cleared as a single entity.

$GdCl_3$-induced macrophage reduction alters VWF catabolism

In an attempt to further explore the contribution of macrophages to the catabolism of VWF in vivo, we treated wild-type mice with $GdCl_3$ (50 mg/kg), an agent that is known to efficiently reduce the number and function of macrophages [24-26]. Indeed, analysis of liver taken from mice 24 h after treatment with $GdCl_3$ revealed a significant 25 % reduction of the number of CD68-positive Kupffer cells (Figure 4A). This reduction was associated with an increase in VWF antigen levels in $GdCl_3$-treated wild-type mice (VWF antigen= 0.72±0.10 U/ml and 1.34±0.40 U/ml for saline- and $GdCl_3$-treated mice, respectively; $p<.05$; Figure 4B). In a second approach, we studied the effect of $GdCl_3$-treatment on the survival of VWF in VWF-deficient mice. Recombinant VWF (5 μg/mouse) was applied to control mice or $GdCl_3$-treated VWF-deficient mice, plasma was collected at indicated time-points and analyzed for the presence of residual VWF antigen. The survival of VWF in control mice was similar as previously reported for untreated VWF-deficient mice (MRT= 2.70±0.35 h) 15. In contrast, the survival of

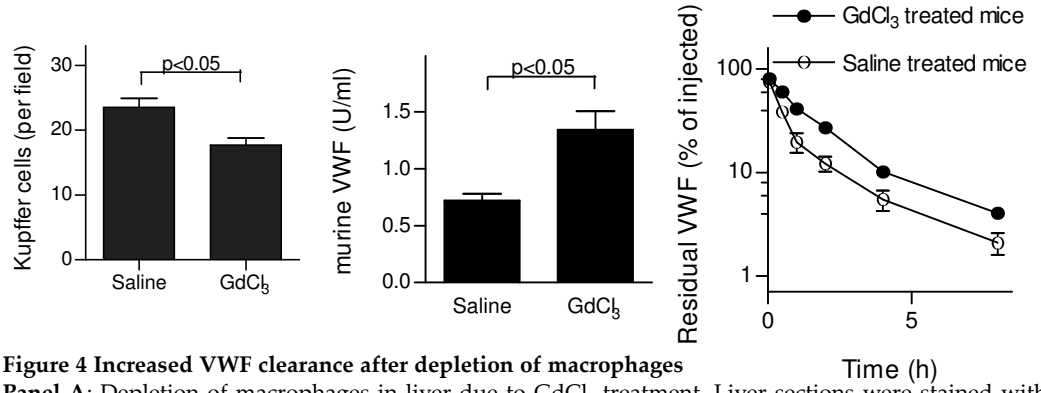


Figure 4 Increased VWF clearance after depletion of macrophages
Panel A; Depletion of macrophages in liver due to $GdCl_3$ treatment. Liver sections were stained with anti- mouse CD68 and analyzed. Macrophage depletion was quantified as the average number Kupffer cells per field from at least 10 randomly selected fields per section. Kupffer cells were identified as CD68 positive cells with an appropriate nuclear morphology and sinusoidal location. Panel B; Wild type mice were treated with $GdCl_3$, 24 hours prior to VWF infusion. After 27 hours VWF antigen levels were determined. As a control mice were pretreated with saline. **Panel C**; VWF deficient mice treated with $GdCl_3$, 24 hours prior to VWF infusion. At the indicated time points VWF antigen levels were measured. The closed circles show VWF antigen levels in VWF deficient mice, pre-treated with $GdCl_3$. The open circles show VWF antigen levels in VWF deficient mice pretreated mice with saline that were used as a control.

VWF in $GdCl_3$-treated mice was dose-dependently prolonged (MRT= 4.53±0.82 h; $p<0.05$ compared to control mice, Figure 4C). Thus, our data point to macrophages as physiological relevant elements in the clearance of VWF.

Human macrophages bind FVIII and VWF in vitro

Having established macrophages as cells contributing to the clearance of VWF and FVIII, the next step was to test whether human macrophages are able to bind both proteins in vitro. Primary monocytes were isolated from human blood and allowed to differentiate into macrophages. Macrophages were incubated with human recombinant FVIII (50 μg/ml) or VWF (10 μg/ml) for 1 h at 4°C to avoid endocytosis. This approach revealed that human macrophages are indeed capable of binding either FVIII or VWF (Figure 5). As is apparent from the co-staining with the macrophage surface marker CD16, binding was limited to the cellular surface as expected (Figure 5). The interaction between macrophages and VWF was investigated in more detail using ^{125}I-radiolabed VWF and THP1-derived macrophages. Binding of ^{125}I-VWF (0-135 μg/ml) to these cells was dose-dependent and saturable (half-maximal binding at 19.7±11.1 μg/ml; Figure 6A). Moreover, binding of radiolabeled VWF (13.5 μg/ml) was markedly inhibited when applied in the presence of increasing amounts of unlabeled VWF (Figure 6B), supporting the specificity of the VWF-macrophage interaction.

Human macrophages rapidly degrade VWF

To test whether human macrophages are also capable of degrading VWF, THP1-cells were incubated with ^{125}I-labeled VWF (13.5 mg/ml) for 1 h at 4°C to allow surface binding. Cells

were then incubated at 37°C to initiate endocytosis. Samples were taken at indicated time-points and analyzed for the presence of TCA-soluble radio-active fragments, which represent degraded VWF protein. Within minutes after inducing endocytosis, VWF degradation products appeared and degradation was virtually complete after 20 min (Figure 6C). Binding and uptake of VWF by macrophages was also visualized using a recombinant VWF-GFP fusion protein. As expected, incubation of THP1-macrophages with VWF-GFP fusion protein (13.5 µg/ml) at 4°C resulted in binding of this protein at the cellular surface (0 min; Figure 6D). Increasing the temperature to 37°C resulted in rapid internalization, as illustrated by the fluorescent signal homogenously present within the cells (2 min; Figure 6D). In time, the intracellular fluorescent signal disappeared, indicating that VWF-GFP protein was degraded within the cells. In conclusion, our data demonstrate that macrophages are able to bind and deliver VWF to their intracellular degradation pathway.

Discussion

VWF plays an essential role in haemostasis facilitating platelet binding to the damaged vessel wall. VWF also circulates in complex with FVIII, thereby increasing FVIII half-life [3,4]. FVIII plasma levels are associated with VWF plasma levels and maintenance of FVIII levels in circulation is dependent on complex formation with VWF. At present relatively little is known concerning the clearance mechanism of VWF [15,27]. Previously, we showed using a mouse model that the majority of VWF was targeted to the liver [15]. Moreover, removal of liver resulted in increased FVIII and VWF levels in a porcine model, leading to increased FVIII half-life [28]. This might suggest that the liver is of importance for the clearance of the VWF/FVIII complex. The impact of liver on FVIII and VWF plasma levels in humans is illustrated in patients suffering from fulminant hepatic failure (FHF). This disease is frequently associated with increased FVIII and VWF levels, despite widespread liver cell death [29-31]. Similar laboratory values have been demonstrated in patients suffering from liver cirrhosis and liver failure. They display highly elevated levels of VWF, which strongly relate to the severity of the disease [32]. Since VWF is predominantly produced outside the liver [33], increased VWF levels in patients with liver failure might be the result of decreased clearance. However, cirrhosis is a very complex disease and is accompanied by large alterations in the haemostatic system. Moreover, VWF from these patients displayed reduced functional properties [32]. Increased VWF levels in these patients may therefore be the result of either an indirect effect on synthesis of VWF or decreased clearance of VWF.

We set up this study to identify cells contributing to the clearance of FVIII and VWF. Recalculation of the data regarding biodistribution experiments [15], showed that also the spleen was particularly efficient in the uptake of VWF (Figure 1). Due to its small size, however, the absolute contribution of spleen to VWF clearance is limited [15]. This is illustrated in patients that underwent a splenectomy. Infusion of DDAVP (resulting in exocytosis of Weibel-Palade bodies, leading to increased VWF plasma levels [34]) in these patients resulted in increased VWF plasma levels. VWF plasma levels declined at an equal rate in asplenic

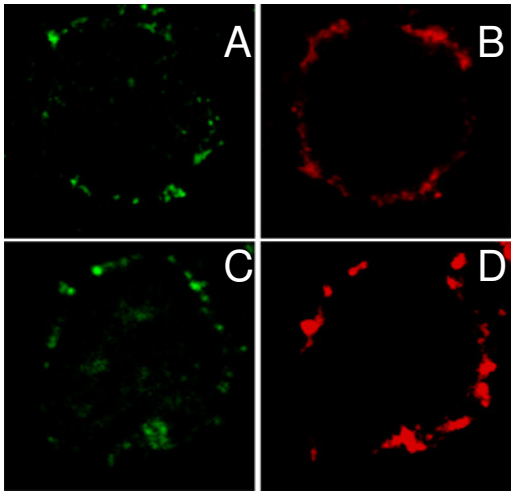
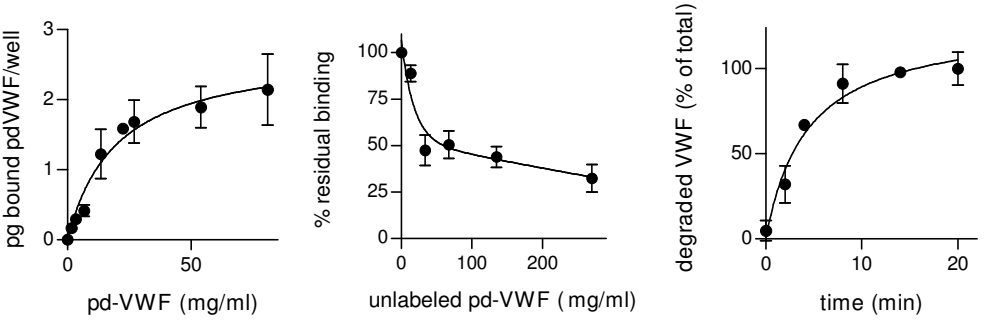


Figure 5 Macrophages bind VWF and FVIII
Binding of VWF and FVIII to primary human macrophages. Monocytes were derived from blood and differentiated into macrophages as described in Methods. Binding of pdVWF and recombinant FVIII was allowed at 4°C. Cells were stained using TRITC conjugated anti-CD16 antibodies and either FITC conjugated anti-FVIII or FITC conjugated anti-VWF antibodies. **Panel A and C** show CD16 labelling of primary human macrophages. **Panel B** shows binding of recombinant FVIII and **Panel D** shows binding of pd-VWF (original magnification 63X).



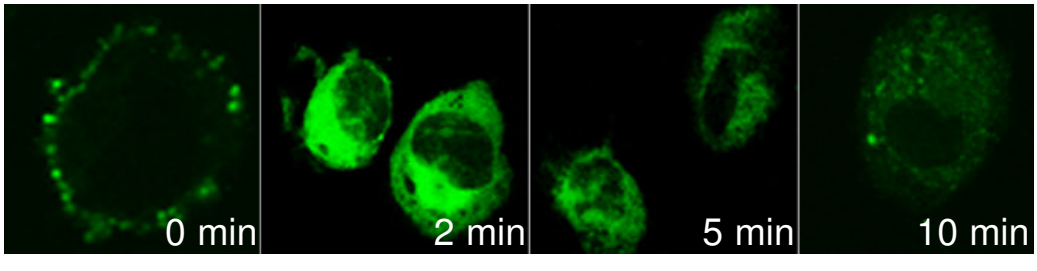


Figure 6 Macrophages bind, internalize and degrade VWF
THP-1 cells were differentiated into macrophages and incubated for 1 hour at 4°C with either radiolabeled pd-VWF (**Panel A, B and C)** or VWF-GFP (**Panel D**). **Panel A**; Bound pd-VWF was determined as described in Methods and pd-VWF binding to macrophages was dose-dependent. **Panel B**; Binding of labelled pd-VWF could be inhibited with an excess of unlabeled pd-VWF. **Panel C**; degradation of radiolabeled pd-VWF by human macrophages. At indicated time intervals. **Panel D;** Images showing binding, uptake and degradation of VWF-GFP by macrophages (original magnification 63X).

patients when compared to normal individuals [35], leading to comparable VWF half-life.

Our data suggest that liver and spleen contain cells capable to remove VWF from the circulation. Both organs are enriched in macrophages and therefore we focussed on the role of these cells in the clearance mechanism of VWF. Indeed, analysis of spleen and liver sections of VWF deficient mice that were infused with VWF, revealed that injected VWF co-localized with macrophages (Figure 2). The interaction between FVIII and VWF is of high affinity [36-39], resulting in a dynamic equilibrium, where a small portion of the FVIII molecules (5-8 %) are not bound to VWF [40,41]. It was therefore interesting to determine targeting of FVIII alone and in complex with VWF. We show here that, like VWF, FVIII appears in spleen and liver, where it co-localizes with macrophages (Figure 3). Several clearance receptors for FVIII have been identified so far [42-45]. It is interesting that a number of FVIII clearance receptors are expressed on macrophages, like LRP-1, mannose receptor and heparan sulfate proteoglycans [46-48]. We also showed that the VWF/FVIII complex appears in spleen and liver after injection in VWF deficient mice (Figure 3). This suggests that both components of the VWF/FVIII complex are targeted to similar cells and cleared as a single entity. Uptake of the complex by macrophages could be facilitated via clearance receptors for FVIII. This is unlikely, since VWF inhibits binding of FVIII to its clearance receptors [44,49-51]. Uptake of the complex by macrophages could also be facilitated by clearance receptors for VWF, that have not been identified so far. Also clearance receptors directed against the VWF/FVIII complex could be present on macrophages. Sarafanov and co-workers have proposed an alternative pathway in which the VWF/FVIII complex is bound to heparin-sulphate proteoglycans at the cellular surface, leading to dissociation of the complex [52]. After dissociation the separate proteins might be internalised by the macrophages.

To examine the contribution of macrophages to VWF plasma levels, wild type and VWF deficient mice were treated with gadolinium chloride ($GdCl_3$). Treatment of mice with $GdCl_3$ resulted in the elimination of about 25% CD68-positive Kupffer cells and a nearly 2-fold increase in VWF plasma levels (Figure 4). Previous studies concerning $GdCl_3$ treatment of rats and other animals showed a decrease in macrophage number and an effect that was more pronounced in liver when compared to spleen [24-26]. Moreover the survival of VWF is also affected. VWF is cleared in a biphasic manner [15]. This might suggest that distinct clearance pathways exist, one with a higher affinity for VWF and responsible for the first phase and one with a lower affinity, responsible for the second phase. After treatment of mice using $GdCl_3$ the initial phase almost disappeared (Figure 4). This implicates that treatment with $GdCl_3$ abolishes the high affinity clearance pathway, leaving the second pathway unaffected.

Our in vitro experiments regarding the cellular uptake of FVIII and VWF confirmed that human macrophages are capable to bind FVIII and VWF (Figure 5). Moreover, macrophages are able to internalise and rapidly degrade VWF. In view of its relatively large size and rapid accumulation in liver, VWF may be removed from the circulation by an active, receptor-dependent process. VWF is a multimeric protein that is composed of identical monomers. All the monomers could contain a binding site for a yet unidentified VWF clearance receptor on macrophages. When macrophages express high numbers of these

receptors on their surface, multiple receptors might be involved in the uptake of VWF. On the other hand, macrophages could contain multiple receptors for VWF multimers. Another striking observation is that internalization and degradation of VWF is completed after only 20 minutes. Protein S is also able to bind to human macrophages. However, internalization and degradation of protein S was completed after 4 hours [53]. This suggests that VWF is internalized by macrophages via a faster mechanism when compared to Protein S.

Considering the impact of VWF on FVIII half-life, it is likely that distinct pathway exists that are responsible for clearance of both components from circulation. However, we showed here that VWF, FVIII and the VWF/FVIII complex are targeted to macrophages in spleen and liver. This might suggest that there is a common clearance pathway for these proteins. On the other hand, these proteins may be targeted to different receptors that are present on similar cells.

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CHAPTER 6

CHAPTER 6

Clearance mechanisms of von Willebrand factor and Factor VIII

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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 b peptide is a hallmark event in the pathogenesis of Alzheimer disease [1], whereas defects in the clearance of lipoprotein particles are associated with familial hypercholesterolemia [2]. 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.

Levels of haemostatic proteins need tight regulation: when levels tend to be too low there is an increased risk of bleeding, whereas there is a predisposition for thrombotic complications when levels are too high. Particular exponents hereof are von Willebrand factor (VWF) and factor VIII (FVIII), both of which are essential for optimal functioning of the haemostatic process. This is well-illustrated by the notion that the genetic or functional deficiency of VWF or FVIII is associated with a severe bleeding tendency, known as von Willebrand disease (VWD) or the X-linked bleeding disorder haemophilia A, respectively [3-5]. On the other hand, several epidemiological studies have revealed an association between the presence of elevated levels of VWF and/or FVIII and thrombotic complications, such as venous thrombosis, stroke and myocardial infarction [6].

A most intriguing aspect that adds to VWF and FVIII is that both proteins circulate in plasma as a tight, non-covalently linked complex. This complex formation is of major physiological relevance, exemplified by markedly reduced FVIII plasma levels that are observed in patients who have no detectable VWF protein (VWD-type 3) or who have a defect in VWF-FVIII complex assembly (e.g. VWD-type 2N) [7-11]. The intricate linkage between VWF and FVIII and its effect on the survival of these proteins has drawn considerable attention, not only because of its impact on haemophilia treatment via FVIII-replacement therapy, but also in view of the finding that abnormal clearance of VWF contributes to the pathogenesis of VWD. Being present as a complex complicates the assessment of how the VWF-FVIII complex or its individual components are removed from the circulation. It is only in the last few years that insight into the molecular basis of VWF-FVIII clearance has been obtained. The present review will address these recent findings.

The life cycle of FVIII

Synthesis of FVIII generates a polypeptide chain of 2351 amino acids, which includes a signal peptide of 19 and a mature protein of 2332 amino acids [12-14]. The molecular structure of FVIII is characterized by a distinct domain structure: A1-a1-A2-a2-B-a3-A3-C1-C2. Due to intracellular proteolytic processing, FVIII is secreted as a heterodimeric protein, that contains a metal-ion linked heavy (A1-a1-A2-a2-B region) and light (a3-A3-C1-C2 region) chain [15,16].

A dominant site of FVIII production seems to be located in the liver, as liver transplantations resulted in sustained, normalized levels of FVIII in a number of cases

concerning haemophilic patients [17,18]. The observations that FVIII mRNA is also present in other organs, such as spleen, lung and kidneys suggest that these tissues may contribute to circulating FVIII levels as well [19-21]. Indeed, recent studies by Jacquemin et al. revealed that human lungs are also capable of producing considerable amounts of FVIII protein [22]. Moreover, the presence of extrahepatic FVIII production has been demonstrated in pigs that underwent total hepatectomy [23]. The cellular origin of FVIII has long been a matter of debate, with reports providing evidence for FVIII production taking place either in hepatocytes or endothelial-like cells. However, more detailed analysis using in situ hybridisation and protein expression studies using isolated cells has let the current opinion to be in favour of liver sinusoidal cells and lung microvascular endothelial cells as main sources of FVIII production [22,24].

Immediately after its release into the circulation, FVIII is caught into a tight non-covalent complex with its carrier protein VWF. Complex formation is crucial for the survival of FVIII in the circulation, and a number of mechanisms have been reported that explain this necessity for complex formation: (i) VWF stabilizes the heterodimeric structure of FVIII [25]; (ii) VWF protects FVIII from proteolytic degradation by phospholipid-binding proteases such as activated protein C (APC) and activated factor X (FXa) [26-28]; (iii) VWF interferes with binding of FVIII to negatively-charged phospholipid-surfaces, which are for example exposed within activated platelets[29,30]; (iv) VWF inhibits binding of FVIII to activated factor IX (FIXa) [31], thereby denying FVIII access to the FX-activating complex, and (v) VWF prevents the cellular uptake of FVIII [32,33], the details of which will be discussed later in this review.

In order to efficiently dissociate from VWF, FVIII is subject to limited proteolysis by thrombin. Thrombin cleavage converts the heterodimeric protein into an instable heterotrimeric derivative, in which the high-affinity VWF binding site is lost. Activated FVIII (FVIIIa) is able to participate in the membrane-bound FX-activating complex (also known as the tenase complex) as a non-enzymatic cofactor, which enhances the proteolytic capacities of FIXa towards the substrate FX. The cofactor activity of FVIIIa in this complex is down regulated by spontaneous dissociation of the instable heterotrimer and/or proteolytic degradation by APC. For more detailed information on FVIII function, we would like to refer to reviews by Fay *et al* [27] and Lenting *et al* [16].

Clearance receptors of FVIII: LDL-receptor related protein-1

Pharmacokinetic studies using therapeutic FVIII preparations have revealed important information concerning the survival of FVIII in haemophiliacs, and how the half-life of these preparations may vary between individuals [34,35]. However, it was only in the end of the 1990's, that reports appeared providing first insight into molecular mechanisms contributing to removal of FVIII from the circulation.

Two groups simultaneously identified the first candidate clearance-receptor for FVIII [33,36]. FVIII was found to interact with modest affinity (25-100 nM) with LDL-receptor related protein-1 (LRP1, also known as a2-macrogobulin receptor or CD91) in systems using purified components. In addition, cells that were deficient for LRP1 proved approximately 50% less

efficient than their LRP1-expressing counterparts in the uptake and degradation of FVIII [16,37,36]. Similar results were obtained by blocking cellular LRP1 with its universal inhibitor Receptor-Associated Protein (RAP). Thus, these data showed that LRP1 is not only able to bind FVIII but also participates in the uptake and delivery of FVIII to intracellular degradation pathways [16,36]. However, an important message that could be easily overlooked from these experiments is that the absence of LRP1 resulted in but a partial inhibition of FVIII degradation. Apparently, LRP1-independent pathways must exist that contribute efficiently to the cellular uptake of FVIII.

A body of evidence is available that supports the in vivo relevance of LRP1 in regulation of FVIII plasma levels. For example, a conditionally induced LRP1 deletion in MX1cre+LRP1$^{flox/flox}$ mice results in increased endogenous levels of FVIII [38]. In addition, the mean residence time (MRT) of intravenously administered FVIII is prolonged 1.5-fold in these mice, from 2.5 hours to 4 hours. From epidemiological studies data are generated, showing that LRP1 modulates FVIII plasma levels also in humans [39-42]. This has become apparent in studies investigating the potential relationship between LRP1 polymorphisms and FVIII plasma levels. So far, two distinct LRP1 polymorphisms (LRP1/D2080N and LRP1/A217V) have been suggested to be associated with increased FVIII plasma levels [40,41]. The underlying mechanism of how these polymorphisms affect FVIII levels remains to be elucidated.

Clearance receptors of FVIII: LDL-receptor family members

Despite proven physiological relevance of LRP1 in FVIII clearance, both in vitro and in vivo experiments point to the involvement of other receptors than LRP1 as well. Indeed, the half-life of FVIII is prolonged to a lesser extent when LRP1 is genetically deleted compared to when LRP1 is blocked via RAP [36,38]. As RAP is also capable of inhibiting other members of the LDL-receptor family, the question arises whether these other family members contribute to FVIII clearance. This question can be answered positively, because Bovenschen and colleagues elegantly showed that the archetype of this family, LDL-receptor (LDLR), is also able to interact with FVIII [43]. Moreover, LDLR seems to regulate FVIII plasma levels in a concerted fashion with LRP1. This cooperation is illustrated by the synergistic increase in FVIII survival upon deletion of both LDLR and LRP1 compared to the genetic deletion of one of them: MRT of FVIII is virtually unaffected by the absence of LDLR, increased 1.5-fold in the absence of LRP1 and increased >4-fold in the absence of both LRP1 and LDLR [43]. As for other members of the LDL-receptor family: no contribution of vLDL-receptor to the *in vivo* clearance of FVIII was found[44], whereas *in vitro* experiments suggest that LRP2/megalin is able to bind FVIII [45]. Its *in vivo* involvement, however, has not yet been reported.

Clearance receptors of FVIII: Proteoglycans and carbohydrate receptors

Receptors that do not belong to the LDL-receptor family have also been suggested to participate in the removal of FVIII from the circulation. Like for many other LRP1-ligands, it seems that heparan-sulfate proteoglycans are a necessary element in the sequestration of FVIII at the cellular surface to facilitate efficient binding to LRP1 [46]. The in vivo relevance of

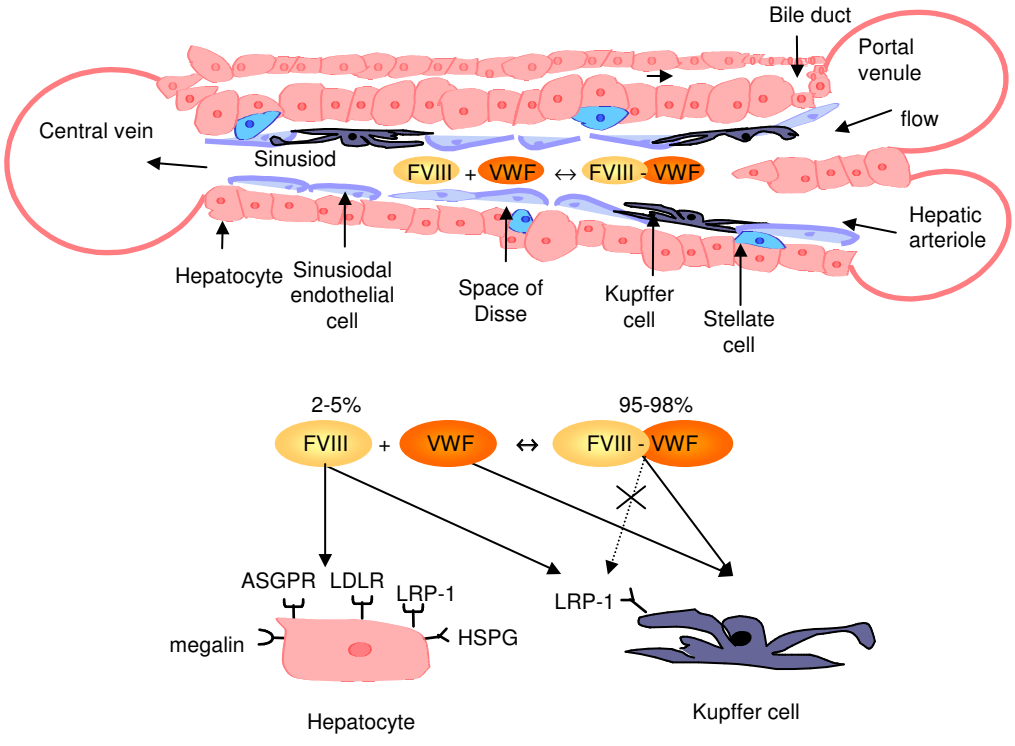

Figure 1. Removal of FVIII, VWF or their complex in liver
Within the liver sinusoid, a number of different cell types can be distinguished: hepatocytes and stellate cells, which are separated from sinusoidal endothelial cells by the Space of Disse. Macrophage-like Kupffer cells are incorporated between the sinusoidal endothelial cells. While circulating, VWF (orange) is in molar excess over FVIII (yellow). The majority of the FVIII molecules (approximately 95-98 %) are found in complex with VWF, while not all VWF molecules are occupied with FVIII. Free FVIII molecules are rapidly cleared via LRP1. Removal of free FVIII forces a constant shift from VWF-bound to free FVIII. LRP1-mediated clearance of unbound FVIII is facilitated by both LDLR and heparan sulphate proteoglycans (HSPG). These receptors have also the potential to internalize FVIII by themselves. Other potential receptors for FVIII are megalin, asialoglycoprotein receptor (ASGPR) and so far unidentified carbohydrate receptors. Each of these receptors is profoundly expressed on hepatocytes, while for some of them expression on macrophages has also been reported. VWF seems to be cleared via a pathway involving Kupffer cells, albeit that the responsible receptor(s) is/are currently unknown. When in complex with VWF, the uptake of FVIII by LRP1 is inhibited.

this step could be demonstrated by co-injection of FVIII with the heparin-blocking agent protamine, which resulted in a modest delay in FVIII clearance. It is of interest to note that besides functioning as a pre-concentrating element at the cell-surface, heparan-sulfate proteoglycans are also able to bind and internalize ligands themselves, albeit at a slower rate than LRP1 [47]. It is possible therefore, that heparan-sulfate proteoglycans can act as independent endocytic receptors for FVIII.

Another example of a recently discovered receptor that binds FVIII is the asialoglycoprotein-receptor [48]. This receptor has already been identified three decades ago to mediate the uptake of proteins exposing terminal β-D-galactose or N-acetyl-D-galactosamine residues [49]. In vitro

binding experiments revealed virtually no binding of B-domainless FVIII to the receptor, suggesting that the FVIII B-domain dominates interactions between receptor and FVIII [48]. In view of the finding that full-length FVIII and B-domainless FVIII have similar half-lives [50], it remains to be assessed what is the precise role of the asialoglycoprotein-receptor in FVIII clearance in vivo. Nevertheless, these data are in support of a so far underestimated role of carbohydrate-recognizing receptors that can contribute to the clearance of FVIII. Given its carbohydrate composition [51-53], candidate receptors in this regard are members of the mannose-receptor family, such as CD206.

Location of LRP1-interactive sites in FVIII

The interaction between FVIII and LRP is characterized by the presence of multiple interactive sites. Within LRP1, at least two distinct sites have been identified, being located in the cysteine-rich repeats clusters II and IV [54]. As for FVIII, four distinct binding sites for LRP1 seem to be present, only three of which have been mapped [33,36,55,56]. One low affinity interactive site is present within the FVIII C2-domain, while high affinity interactive sites are located in the A2 and A3 domain, spanning residues Arg484-Phe509 and Glu1811-Lys1818, respectively. Intriguingly, each of these regions are known to be of importance for FVIII biology, as they not only comprise interactive sites for FIXa, but are also well-known targets for inhibitory antibodies that might develop during haemophilia A treatment. It is possible that this overlap is just coincidence, as these regions are well exposed at the surface of the FVIII molecule and therefore fulfil criteria for interactive sites and inhibitor epitopes. Alternatively, recombinant LRP1 fragments are potent inhibitors of FVIIIa/FIXa-mediated FXa generation [57], which may point to a potential regulatory role of LRP1 in the down regulation of the tenase complex.

Exposure of LRP1-interactive sites in FVIII

One intriguing issue that remains relates to the accessibility of the respective LRP1 interactive sites under physiological conditions. In their first report on the interaction between FVIII and LRP1, Saenko and coworkers described the binding of the intact FVIII heavy chain and the FVIII A2 domain to LRP1 [36]. However, binding of the intact FVIII heavy chain to LRP1 was not observed in a study by one of us [33]. It has recently been confirmed that the intact heavy chain binds poorly, if at all, to LRP1 [58]. This lack of interaction explains why site-directed mutagenesis of the LRP1 binding site in the A2 domain did not result in improved pharmacokinetic parameters when mutated FVIII molecules were tested in mice and rats [59]. In fact, the LRP1-interactive site within the A2 domain becomes exposed exclusively after proteolysis of the heavy chain into its constituents, the A1 and A2 domain [58]. This suggests that the LRP1-FVIII A2 domain interaction may be part of down-regulatory mechanisms that lead to inactivation at and/or removal of FVIIIa from the cellular surface.

Interestingly, exposure of LRP1 binding sites located in FVIII light chain is independent of proteolysis. Intact light chain as well as its thrombin- or FXa-cleaved derivatives display similar efficiency in binding to LRP1 or recombinant fragments thereof

[33,58]. However, exposure of LRP1 interactive sites is subject to regulation, albeit not via proteolysis. The main player in this regard is VWF, which interacts with the N- and C-terminal ends of FVIII light chain [60]. As mentioned before, this interaction is known to prevent the interaction between FVIII light chain and phospholipids as well as the interaction between FVIII light chain and FIXa. In view of the overlap of interactive sites for FIXa, phospholipids and LRP1, it is not surprising that VWF also interferes with the interaction between FVIII light chain and LRP1. VWF does not only prevent binding of FVIII to LRP1 in a system using purified proteins, but also in cellular degradation assays [32,33]. Potential mechanisms that allow VWF to interfere with the FVIII-LRP1 interaction include direct competition for binding to the C2 domain, steric hindrance and alteration of the FVIII conformation so that LRP1 cannot bind. Of course, proteolytic activation of FVIII into FVIIIa results in loss of high-affinity binding to VWF and thereby indirectly exposes LRP1 binding sites within FVIII light chain.

Consequences of regulated exposure of LRP1-interactive sites

By identifying the abovementioned mechanisms as regulators of the FVIII-LRP1 interaction, it is tempting to jump to the conclusion that FVIII survival is independent of LRP1 in the normal circulation, since FVIII circulates in tight complex with VWF (thus protecting LRP1 binding sites in the light chain) with an intact heavy chain (thus with the LRP1 binding site in the A2 domain being encrypted). However, this conclusion is in opposition with the in vivo observations in which deletion or blockage of LRP1 in mice with normal VWF leads to increased FVIII levels and prolongation of FVIII survival. Thus, how is it possible that LRP1 contributes to FVIII clearance while FVIII is tightly bound to VWF?

A first option is that the VWF/FVIII complex itself is cleared in vivo as a single entity via an LRP1-dependent mechanism. It is important to stress in this regard that VWF itself does not interact with LRP1 [33], leaving only the option that LRP1 indirectly affects VWF clearance. Such mechanism would provide a rationale for the observation that VWF levels are slightly, but significantly increased in mice with a conditional LRP1-deletion [38]. Clearance studies monitoring the survival of VWF in these mice are needed to address this possibility.
Sarafanov and coworkers have proposed an alternative pathway in which the VWF/FVIII complex is bound to heparin-sulphate proteoglycans at the cellular surface, leading to dissociation of the complex [46]. Once dissociated, FVIII is transferred to LRP1, whereas VWF is released into the circulation. The applicability of this pathway is probably limited to but a subset of cell types, because VWF completely blocks FVIII uptake in several cell types that have been tested in vitro [32,33]. This potential limitation is in line with the modest effect of blocking heparan-sulphate proteoglycans with protamine on FVIII clearance [46].

A third aspect that deserves to be considered relates to the formation of the FVIII/VWF complex itself. The interaction between FVIII and VWF is of high affinity, and in several studies this affinity has been calculated to be less than 1 nM [61-63]. Kinetic analysis revealed that complex formation represents a highly dynamic equilibrium, characterized by high association and dissociation rate constants (k_{on} ~2x106 mol-1 s^{-1} and k_{off} ~1x10-3 s^{-1}) [61,64,65]. As a consequence of this dynamic equilibrium, a small but significant portion of the

FVIII molecules (5-8 %) are not bound to VWF, but circulate as a free protein [65,66]. As a free protein, LRP1 binding sites in FVIII light chain are not protected anymore by VWF, allowing LRP1-mediated clearance. Since FVIII is cleared 4-6 fold more rapidly as a free protein compared to when it is bound to VWF [9], the loss of free FVIII is compensated by a continuous flow of VWF-bound to free FVIII in order to maintain the balance. But what happens then with the remaining, VWF-bound bulk (92-95 %) of the FVIII molecules? Most likely, distinct pathways are involved in the clearance of FVIII versus the FVIII/VWF complex. It seems conceivable that VWF-bound FVIII follows VWF in its clearance pathway.

Life cycle of VWF

VWF is a multimeric glycoprotein that is produced in endothelial cells and megakaryocytes [67]. Megakaryocytic production is responsible for the presence of VWF in the a-granules of platelets, whereas endothelial cells are the primary source of VWF that is found in the subendothelial matrix and in plasma. VWF is synthesized as a single-chain pre-pro-polypeptide, in which a number of domainal structures can be distinguished: D1-D2-D'-D3-A1-A2-A3-D4-B1-B2-B3-C1-C2-CK [68]. During synthesis, VWF undergoes extensive processing, including carboxy-terminal dimerization and proteolytic separation of the propeptide (D1-D2 domains) from the mature subunit that comprises the remaining domains. The main functions of the propeptide are to target VWF to the Weibel-Palade bodies and to facilitate amino-terminal coupling of mature VWF subunits into multimers. The latter process generates a heterogeneous pool of VWF multimers, the molecular mass of which may range from $0.5x10^6$ Da to over $10x10^6$ Da. Posttranslational modification further involves the attachment of N- and O-linked carbohydrate side chains.

Several physiological functions have been ascribed to VWF. As discussed extensively above, VWF functions as a carrier protein for FVIII. VWF also plays a dominant role in the recruitment of platelets to sites of vascular injury, particularly under conditions of rapidly flowing blood [69]. Platelet recruitment by VWF involves the initial step of platelet adhesion via simultaneous interactions with subendothelial collagen and the platelet glycoprotein (Gp)-Ib-IX-V complex. In a secondary step, VWF mediates inter-platelet bridging via RGD-dependent interactions with αIIbβ3, allowing the formation of platelet aggregates.

Apart from its role in thrombus formation and the protection of FVIII, 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 [70,71]. These organelles are not only a storage-compartment of ultra-large VWF multimers, but also for a variety of other proteins, such as P-selectin, interleukin-8, angiopoietin-2 and osteoprotegerin [72,73]. Second, the presence of VWF has a suppressing effect on the metastatic potential of tumour cells [74]. Third, VWF comprises binding sites for Staphylococcal aureus-surface proteins (Protein A), which may facilitate intravascular colonisation by these pathogens [75-77]. Further, VWF may promote the proliferation of smooth muscle cells [78]. Finally, we have recently reported on the ability of VWF to act as an adhesive surface for leukocytes [79]. VWF-leukocyte interactions involve P-selectin glycoprotein ligand 1

(PSGL-1) as well as the family of β2-integrins.

Clearance of VWF

Although equipped with the capacity to play part in various (patho)-physiological processes, it is in the line of reason to assume that the majority of VWF molecules that are produced will never participate in any of these processes, simply because they persist in the circulation but for a short period of time. On average, the half-life of intravenously administered VWF antigen in VWD-type 3 patients is about 15 hours, albeit that inter-individual differences in VWF survival have been reported [80-82]. Also the survival of endogenous VWF may differ markedly between persons, as is apparent from studies using desmopressin treatment [83]. This agent induces the release of VWF stored in Weibel-Palade bodies, resulting in a temporary rise in VWF levels [84]. The time it takes for VWF to return to base-line levels may therefore be used to calculate the half-life of endogenous VWF. Simultaneously with VWF multimers, also VWF propeptide is released from the Weibel-Palade bodies, in a 1:1 molar ratio with mature VWF. However, the survival of propeptide is 3-4 fold shorter compared to VWF, resulting in a distinct propeptide/VWF ratio under steady state conditions [85].

A number of parameters have been identified that seem to be responsible for differences in VWF half-life, although it should be mentioned that some of them may also influence the extent of VWF production. In the following paragraphs, a number of these parameters will be discussed.

The effect of glycosylation on VWF clearance: Blood group determinants

The average plasma level of VWF is ~10 μg/ml, although a broad range between individuals has been reported (ranging from 40% to 240% of the population mean) [86]. One dominant factor that influences VWF plasma levels is its glycosylation profile [87]. VWF is a glycosylated protein that contains 10 sites for O-linked and 12 sites for N-linked glycosylation. It has been demonstrated that part of the N-linked sugars contains ABO-blood group determinants [88,89], which are absent on the O-linked sugars as well as within the propeptide [90,91]. Interestingly, the nature of the ABO-determinant is strongly related to VWF levels: the average VWF concentrations are approximately 25% lower in persons with blood group O than those in non-O individuals (reviewed in [92]). Levels are even more reduced in persons with the Bombay-phenotype, who lack expression of ABO-antigens [93].

How to explain the lower levels of VWF in those who have blood group O? A number of possibilities have been proposed, such as impaired biosynthesis or secretion of VWF in blood group O-persons. However, this possibility has proven difficult to be addressed in vitro, because VWF synthesized in vitro differs significantly with regard to its glycan structures compared to VWF that circulates in plasma [94,95]. In vivo analysis has not revealed data that support an effect of the ABO-determinants on synthesis and/or secretion: the total increase in VWF upon desmopressin treatment is not significantly different for group O and non-O individuals [96]. A more likely explanation for reduced VWF levels in blood group O-persons relates to a decreased survival. First, one would expect the propeptide/VWF ratios to

be increased in the blood group O-population, as their VWF is cleared more rapidly, whereas their propeptide is not. Indeed, in two recent studies by Haberichter *et al*. and Nossent *et al*., ratios were consistently found to be higher in the O-group compared to the non-O-group [90,97]. Nossent *et al.* further used a mathematical approach assuming a similar clearance rate of propeptide amongst individuals, allowing them to estimate the half-life of endogenous VWF. From these calculations, the half-life of endogenously produced VWF was found to be 2 hours longer in the non-O population compared to the O-population. Additional evidence that blood group O-determinants on VWF are associated with increased clearance is provided by observations that infused FVIII (either plasma-derived or recombinant) disappears more rapidly from the circulation in blood-group O than in non-O haemophilia A patients [98]. Of course, this evidence seems only valid assuming that FVIII follows endogenous VWF in its clearance pathway.

An interesting consequence of the blood-group dependent clearance rate is that it would be attractive to develop plasma-derived VWF concentrates that are enriched in non-O containing VWF molecules. Moreover, in view of the development of a recombinant-derived VWF therapeutic preparation [99], it is of importance to investigate how the non-human glycosylation profile affects the survival of VWF in humans.

Apart from the ABO-blood group effect, also other blood group-dependent modifications of the glycosylation profile may affect VWF plasma levels, with particular reference to the Secretor system. 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 [100]. 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

Another direct link between VWF glycosylation and clearance is evident from studies employing mice deficient for ST3Gal-IV. This enzyme mediates the attachment of sialyl-groups to terminal galactose-residues. In ST3Gal-IV deficient mice, the half-life of endogenous VWF is reduced two-fold 101. In addition, in a patient group referred to the hospital for real or suspected bleeding disorder, reduced ST3Gal-IV-mediated sialylation was observed to be associated with reduced VWF plasma levels [101]. Thus, sialylation is an important measure to prevent premature clearance via receptors that recognize non-sialylated terminal galactose-residues, such as the hepatic asialoglycoprotein-receptor. Further support for the protective effect of sialyl-groups comes from studies by Sodetz *et al*., who demonstrated that enzymatic removal of sialyl-groups reduces half-life of VWF in rabbits from 240 min to 5 min [102].

The importance of the nature of the terminal carbohydrate-residue is most strikingly illustrated by RIIIS/J mice. This mouse strain is characterized by VWF levels that are several-fold lower compared to other mouse strains [103]. Via a crossbreeding approach, it was demonstrated that the gene responsible for these low VWF levels was distinct from the VWF

gene [104]. More detailed studies identified this VWF-modifier gene as being *Galgt2* (currently renamed as *B4galnt2*), which encodes an N-acetylgalactosaminyltransferase [42]. The activity of this enzyme determines the expression of an oligosaccharide specific for a murine T cell subpopulation [105]. The human equivalent of this enzyme is responsible for expression of the blood-group antigen Sda. Murine expression of this glycosyltransferase is limited to intestine and kidney. In RIIIS/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 [106]. Upon secretion into plasma, this form of VWF is readily recognized by the asialoglycoprotein-receptor and subject to rapid clearance.

Both 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

As mentioned, VWF is also subject to O-linked glycosylation, and currently little is known concerning its effect on VWF clearance. Stoddart and colleagues demonstrated that recombinant VWF lacking all O-linked carbohydrates has a reduced half-life when administered in rats [107]. This may indicate that O-linked sugars have a protective effect on VWF survival. Alternatively, a complete suppression of O-linked glycosylation during synthesis may seriously compromise correct folding of the protein. It seems conceivable therefore that the reduced half-life originates from suboptimal folding of VWF.

The main O-linked glycan moiety (at least 70%) is composed of the sialylated tumor-associated T-antigen [91]. This structure consists of the disaccharide galactose(β1-3)N-acetylgalactosamine and is sialylated through capping with two N-acetylneuramic acid residues (i.e. NeuAc(α2-3)Gal(β1-3)[NeuAc(α2-6)]GalNAc). To explore the connection between O-linked glycosylation and the regulation of VWF levels, we have recently developed an assay to specifically quantify the extent of glycosylation with sialylated T-antigen. In a large group of healthy individuals we observed a negative relationship between the presence of the sialylated T-antigen and VWF levels; thus the more glycosylation, the lower the levels of VWF [108]. This association was most pronounced under pathological conditions, where VWF levels were outside the normal range. One such example involves liver cirrhosis patients, who have levels of VWF that may be increased up to 2000 % of normal. In these patients, the extent of O-linked sialylated T-antigen was significantly reduced by more than 2-fold on average. As for patients with reduced levels of VWF (patients with VWD-type 1 in our study), the opposite was found in that glycosylation with sialylated T-antigen was increased up to 3.6-fold .

It remains to be investigated whether differences in O-linked glycosylation modulate the clearance rate. However, there is one observation, which is in support of this possibility. We observed that the propeptide/mature VWF ratio was increased 2-fold in the VWD-type 1 patient group compared to normal individuals. Moreover, there was a strong linear correlation between this ratio and the extent of O-linked glycosylation [108], suggesting a

decreased VWF survival in those patients who were characterized by increased glycosylation with sialylated T-antigen.

The effect of ADAMTS13 on VWF clearance

When freshly released from storage-granules, part of the VWF molecules can be classified as ultra- or unusually large (UL)-multimers [109,110], a subclass of molecules that are able to interact spontaneously with the platelet GpIb-IX-V complex [111]. To avoid the formation of VWF-platelet aggregates that may occlude the circulation, these UL-multimers are subject to proteolysis [112]. 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 [113,114]. The gene encoding this protease is closely located within chromosome region 9*q*34, which is remarkably close to the ABO-locus (distance of about 140,000 base pairs) [115]. This chromosome region has been identified as an important genetic determinant of VWF levels, and it has been speculated therefore that ADAMTS13 cleavage may lead to enhanced clearance of VWF [116]. However, the experimental data that are currently available seem to oppose the validity of this hypothesis. First, basal VWF levels are similar in normal and ADAMTS13-deficient mice (ie. provided that wt- and deficient mice of the same genetic background are compared) [117]. One would expect levels to be increased in ADAMTS13-deficient mice if proteolysis by this protease would enhance the disappearance from the circulation. Second, when wt-VWF and a variant containing a type 2A mutation (making VWF more susceptible to ADAMTS13-cleavage) were compared for their survival in a rat-model, there appeared to be no difference in clearance rate [107]. It is unlikely therefore, that ADAMTS13 cleavage results in an accelerated clearance of the protein.

Effect of mutations on VWF clearance

VWD is classified into three major types: quantitative deficiencies of the protein are defined as type 1 (mild deficiency) or type 3 (severe deficiency), whereas qualitative effects are categorized as type 2 [118]. A large array of mutations in the VWF gene has been associated with VWD. Previous studies have revealed considerable insight into how these mutations may impair biosynthesis and/or secretion, thereby explaining low VWF levels in these patients. In recent years, however, it has become clear that also increased clearance may contribute to the pathogenesis of VWD. Casanato and coworkers provided a first report in this regard, in which they described a study concerning the effect of desmopressin-treatment in VWD patients with the so-called Vicenza subtype [119]. As expected, desmopressin-treatment resulted in a concordant increase in VWF levels in these patients that reached near normal levels. However, this freshly released VWF disappeared much more rapidly from the circulation compared to that in a control group. These data suggested that the mutation in the mature VWF subunit (i.e. Arg 1205 to His within the D3 domain) was associated with an increased clearance rate [119]. Of note, from that report it remained unclear whether the decreased survival originated from the Arg1205His replacement, an additional gene mutation (only part of the VWF coding

sequence was analysed) or an eventual defect in a receptor involved in the cellular uptake of VWF. To demonstrate a direct relationship between this particular mutation and the observed increased clearance, we have constructed a recombinant variant comprising Arg1205His, and tested the purified recombinant protein in VWF-deficient mice for its survival [120]. This analysis revealed that the MRT of this mutant in VWF-deficient mice was 0.3 h, compared to 2.8 h for wt-VWF, clearly demonstrating that the survival of the mutated protein is severely reduced.

Since the description of the Arg1205His mutation as a cause for decreased survival of VWF, a number of other mutations have been described as well. These include Cys1130Phe and Cys1149Arg that are located in the D3 domain and the CK-domain residue Cys2671Tyr, the recombinant derivatives of which have been tested in our experimental mouse model [121]. Each of these mutants displayed reduced survival, albeit to a lesser extent compared to VWF/Arg1205His (i.e. MRT's of approximately 0.7 h, which is about 4-fold shorter than for wt-VWF, but 2.3-fold longer than VWF/Arg1205His). The association between the presence of these mutations and reduced survival could be confirmed in patients harbouring these mutations. First, their endogenous VWF disappeared 4.5-fold quicker upon desmopressin compared to a group of normal controls [121]. Second, these patients were characterized by an increased propeptide/VWF ratio, which is a surrogate marker for increased clearance.

The approach to monitor propeptide/VWF ratio has been used in a more extensive manner by Haberichter and colleagues, who analysed 4 families suspected for VWD-type 1 [97]. Within these families, all affected family members were having a propeptide/VWF ratio that was outside the normal range. More detailed analysis was subsequently performed to monitor VWF survival after desmopressin-treatment. As expected, in these patients a strongly decreased survival of endogenous VWF was observed. In search for the mutation, the VWF gene of these patients has been sequenced, revealing that two families had a Tyr1144 to Gly replacement in the D3 domain, and two families a Ser1179 to Phe mutation in the D4 domain. In total, 6 different mutations have now been identified that are associated with increased clearance, explaining at least in part the low VWF levels in these patients. This number suggests that increased clearance is a more common phenomenon in the pathogenesis of VWD than previously anticipated. The common occurrence has been confirmed in a study by Brown *et al.*, who compared the survival of VWF after desmopressin-treatment in a group of VWD-type 1 patients to that of a group of haemophilia A patients [96]. The average survival of VWF in VWD-type 1 patients was indeed significantly lower than in haemophilia A patients, although the report did not allow assessment of the individual data. It is to be expected that additional mutations associated with increased VWF clearance will be identified.

Molecular basis of VWF clearance

How is it possible that so many different mutations are associated with increased clearance? No clear answer to this question is yet available. Most of the mutations have been analysed for their effect on VWF function, and no common defect was observed [120,121]. This suggests that structural consequences of the mutations differ per mutation, and that there is apparently no

obvious structural denominator that renders the mutant proteins with decreased survival.

To gain insight in the relative contribution of VWF regions to the clearance of this molecule, we have compared the in vivo survival of various recombinant VWF variants [120]. A fragment comprising the A1-A2-A3 domains displayed a similar survival to that of fully multimerized VWF. When this fragment is extended C-terminally with the D4-CK region, both initial recovery (ie. 3 min after injection) and MRT are reduced about 2-fold in comparison with wt-VWF. In contrast, when the A1-A2-A3 fragment is extended N-terminally with the VWF-D'D3 domains, MRT is increased from 2.8 to 4.5 h. Moreover, the D'-D3 region itself displays a similar prolongation of its survival (MRT=5.9 h). Based on these observations, it seems fair to draw the following conclusions: (i) various regions within the VWF molecule contribute to the clearance process, (ii) the A1-A2-A3 region contains a receptor-binding site, (iii) the regions D4-CK and D'-D3 comprise receptor-binding sites and/or regulatory sites that permit the enhanced or reduced exposure of the receptor-binding site within the A1-A2-A3 region. It is of further importance to note that four out of six mutations identified so far are located within the D'-D3 region of the VWF molecule. This may suggest that mutation-dependent alterations in this region increase the susceptibility of VWF to increased clearance more than in other parts of the VWF molecule.

Cells involved in VWF clearance

In comparison to FVIII, little information is available on the identity of cells and receptors that mediate clearance of VWF. Various mechanisms are known that provide the basis for the removal of proteins from the circulation: interactions with one or more specific endocytic receptors; proteolysis; secretion via kidneys; or extravasation. In case of VWF, it is unlikely that (ADAMST13-mediated) proteolysis contributes to a significant extent. In the various experimental models, no proteolytic conversion of high VWF multimers into smaller derivatives has been observed [107,120]. In view of its molecular size, it is further improbable that VWF is secreted via the kidneys or that VWF passes spontaneously from the circulation through the endothelium to the extravascular space. In contrast, its structural diversity may allow VWF to interact with multiple receptors, thereby favouring clearance of VWF to involve a receptor-mediated process.

In search for the cellular basis of VWF clearance, we have recently monitored biodistribution of intravenously injected VWF. We observed that the bulk of protein was targeted to the liver, whereas little protein was found in other organs [120]. However, when expressed in relative terms, spleen was as efficient as liver in the uptake of VWF. Spleen and liver have in common that they are enriched in sinusoidal endothelial cells and macrophages (Kupffer cells in liver). Since macrophages are able to rapidly internalize large particles, they are attractive candidates to function as cells that mediate VWF uptake. In a preliminary study, we indeed observed that Gadoliniumchloride-mediated depletion of macrophages resulted in a 2-fold increase of endogenous VWF levels (Chapter 5). Moreover, the survival of VWF administered via tail-vein injection was significantly prolonged (MRT = 4.5 h compared to 2.7 h in control mice). Another indication that macrophages may contribute to the uptake of VWF

is apparent from the immuno-histochemical analysis of spleen tissue isolated from VWF-deficient mice injected with VWF. VWF was clearly present when detected using polyclonal anti-VWF antibodies. In particular, VWF was detected in the marginal zone, a region enriched in macrophages that borders the area known as white pulp. Indeed, comparison of images stained for VWF and macrophages revealed a considerable overlap, suggesting accumulation of VWF in macrophages.

Receptors involved in VWF clearance

So far, no endocytic receptors have been identified that mediate the removal of VWF from the circulation, with the exception of the asialoglycoprotein-receptor. However, the reactivity of this receptor towards VWF is limited to those cases of hypo-sialylation or altered glycosylation of VWF [101,106]. Nevertheless, considering the strong influence of the glycosylation-profile on VWF clearance, it seems conceivable that other carbohydrate-recognizing components play an important role in the removal of VWF from the circulation. Alternatively, we have recently reported that VWF may act as an adhesive surface for leukocytes, the adhesion of which to VWF is mediated by β2-integrins [79]. In particular, αMβ2 integrin (also known as MAC-1 or CR3) is well-known for its involvement in the uptake of microbes and proteins such as fibrinogen by macrophages, and is therefore an attractive candidate to serve as an endocytic receptor for VWF. It is obvious that other candidates may exist, and it is the challenge for the near future to identify those that mediate the cellular uptake of VWF.

Conclusion

In the past decade important progress has been made in our understanding of the mechanisms that control circulating levels of FVIII and VWF. Of course, years of study will be needed to reveal every aspect in full detail and essential data on for example the identity of VWF-clearance receptors is still lacking. Nevertheless, the current knowledge is already promising in that it may find application in the design of recombinant variants that display prolonged survival. However, one should remain careful in the use of such therapeutic preparations. For instance, how will such modulations affect the cellular targeting of FVIII, VWF or its complex? It is not unthinkable that these modulations could result in a more efficient targeting to antigen-presenting cells, thus provoking an unwanted stronger immune response than with the currently available preparations. Apart from potential applications in the treatment of haemophilia, our understanding of the clearance mechanisms is of relevance for the diagnosis of certain VWD subtypes. Increased clearance of VWF is an aspect that is now recognized to be an important determinant of low VWF levels in VWD-type 1. Haberichter *et al*. have proposed to classify this phenotype as VWD-type IC (1-clearance), a proposal that is supported by us. Of course, increased clearance is not selective for VWF. We have for instance reported that low levels of protein S-Ser460Pro (Heerlen polymorphism) may be explained by a 4-fold reduced survival of this mutant [122]. Whether or not FVIII mutations exist which are associated with increased clearance, remains to be investigated.

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CHAPTER 7

CHAPTER 7

Staphylococcus aureus Protein A binding to von Willebrand factor A1 domain is mediated by conserved IgG binding regions

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Abstract

Protein A (Spa) is a surface-associated protein of *Staphylococcus aureus* best known for its ability to bind to the Fc region of IgG. Spa also binds strongly to the Fab region of the immunoglobulins bearing V_H3 heavy chains and to von Willebrand factor (VWF). Previous studies have suggested that the protein A-VWF interaction is important in *S. aureus* adherence to platelets under conditions of shear stress. We demonstrate that Spa expression is sufficient for adherence of bacteria to immobilized VWF under low fluid shear. The full-length recombinant Ig-binding region of protein A, Spa-EDABC, fused to glutathione-S-transferase (GST), bound recombinant VWF in a dose-dependent and saturable fashion with half-maximal binding of about 30 nM in immunosorbent assays. Full-length (FL)-Spa did not bind recombinant VWF A3 domain but displayed binding to recombinant VWF domains A1 and D'-D3 (half-maximal binding at 100 nM and 250 nM, respectively). Each recombinant Protein A Ig-binding domain bound to the A1 domain in a similar manner to the FL-Spa molecule (half-maximal binding 100 nM). Amino acid substitutions were introduced in the GST-SpaD protein at sites known to be involved in IgG Fc or in V_H3-Fab binding. Mutants altered in residues that recognized IgG Fc but not those that recognized V_H3 Fab had reduced binding to VWF-A1 and D'-D3. This indicated that both VWF regions recognized a region on helices I and II that overlapped the IgG Fc binding site.

Introduction

Staphylococcus aureus is a commensal of moist squamous epithelial surfaces, primarily in the anterior nares, where it permanently colonizes 20% of the population and transiently colonizes another 60% [1]. It can cause superficial skin infections such as abscesses and impetigo, and more serious invasive infections such as osteomyelitis, septic arthritis and endocarditis. Indeed, *S. aureus* is the leading cause of infective endocarditis where the bacterium can initiate infection of previously undamaged heart valves [2].

S. aureus has the ability to express a number of cell wall-anchored surface proteins that bind to plasma proteins or to components of the extracellular matrix. This facilitates evasion of immune responses, colonization of damaged tissue and adhesion to host cells and to platelets [3-5]. The interaction between *S. aureus* and platelets is a critical step in *S. aureus*-induced endocarditis. It has been demonstrated previously that the plasma proteins fibrinogen and fibronectin can act as bridges between bacterial cells and the platelet intergrin GPIIb/IIIa on resting platelets, through interactions with staphylococcal surface proteins clumping factor A and fibronectin binding proteins [6-9]. In each case, antibodies specific to the surface protein are also required to form a bridge to the platelet FcγRIIa receptor. This can lead to infective vegetations in the vascular endothelium.

Protein A (Spa) is a major surface protein of *S. aureus*. It comprises 4 or 5 homologous repeat domains of 56-61 residues followed by a polymorphic variable repeat region X_r and a conserved region X_c, which includes a cell-wall attachment sequence [10,11]. Structural analysis of a single Spa domain revealed that it is composed of a 3 helical bundle. The solved structures of Spa domains in complex with IgG Fc and with a V_H3-derived IgM Fab demonstrate how different parts of the Spa repeat are involved in the two interactions. Indeed, it is possible for a single Spa domain to bind each ligand simultaneously [12-14].

Spa is known to bind human von Willebrand factor (VWF), a protein that is essential for haemostasis, with an affinity of 15 nM as measured by surface plasmon resonance using full length recombinant protein A and VWF that had been purified from plasma. This interaction was shown to occur in the presence of physiological IgG concentrations [15]. Heritable defects in VWF result in von Willebrand's disease, a common bleeding disorder, symptoms of which can mirror severe haemophilia. The main function of VWF is to capture platelets by binding to the platelet receptor GPIb-α and immobilise them at the site of damage to a blood vessel and to stimulate the formation of a blood clot. The VWF protein consists of four types of repeat domain A, B, C and D. Domains are arranged in the sequence D'-D3-A1-A2-A3-D4-B1-B2-B3-C1-C2-CK in the mature protein (for review, see [16]). The crystal structure of the recombinant A1 domain in complex with platelet glycoprotein GpIbα has been solved [17-19]. Binding of circulating VWF to the ligands such as collagen in exposed subendothelial matrix of damaged blood vessels under high shear-stress stimulates a conformational change which promotes immobilized VWF binding to GpIbα on platelets [20-22]. Circulating platelets are captured and activated, stimulating the formation of a thrombus [23,24].

The ability of *S. aureus* to bind VWF could contribute to the adherence of the bacterium to platelets or to damaged blood vessels. By studying a Spa-deficient mutant of *S. aureus* it was shown that the Spa-VWF interaction is necessary for efficient recruitment of *S. aureus* by platelets under high shear stress in whole blood [25]. Also fluid-shear adhesion experiments suggested that VWF binding to Spa can promote adherence of circulating *S. aureus* cells to immobilised collagen [26]. In this study we set out to analyze the interaction between Spa and VWF. We demonstrate, using *Lactococcus lactis* as a surrogate host for protein expression, that Spa is sufficient for adherence of bacteria to immobilized VWF under low shear conditions. We used recombinant Spa and VWF truncates to identify and characterize the domain(s) in each protein that are involved in binding and refined the VWF binding domain in protein A by site-directed mutagenesis.

Materials and Methods

Bacterial strains and growth conditions

DNA cloning was performed in *Escherichia coli* XL1-Blue (Qiagen, Madison, Wis., USA). TOPP3 (Stratagene, La Jolla, CA, USA) or M15(pREP4) (Qiagen) were used for expression of recombinant proteins. *E. coli* was routinely grown in L-broth at 37° C with shaking at 200 rpm. *L. lactis*::pKS80 and *L. lactis* pKS80::spa$^+$ were grown in M17 medium containing 0.5 % (v/v) Glucose. Ampicillin (100 μg/mL), kanamycin (50 μg/mL) and erythromycin (10 μg/mL) were incorporated into the media where appropriate.

DNA constructions

Fragments of the region of *spa* encoding the extracellular immunoglobulin-binding domains were isolated and cloned as follows; plasmid pSPA7235 27 containing the entire coding region of the *spa* gene of *Staphylococcus aureus* 8325-4 was used as a template for amplification of the five- and single-domain repeats of *spa*. Oligonucleotides were designed to each individual domain to allow amplification of individual protein A repeats (Table 1). Cross reaction of primers due to high sequence homology of the repeats was overcome by digestion of the *spa* template with unique restriction endonucleases prior to single domain amplifications. Restriction sites were incorporated at the 5' ends of the primers to facilitate directional cloning. PCR amplification was carried out in a DNA thermal cycler (Techne, Cambridge, U.K.) with *Pfu* DNA-polymerase (Stratagene). Reactions were carried out with a 1-min denaturation step at 94 °C, a 1-min annealing step at 55 °C, and elongation at 72 °C for 1-2 min depending on the length of the desired PCR product. This standard cycle was repeated 30 times followed by incubation at 72 °C for 10 min. PCR products were purified using the High Pure™ PCR product purification kit (Roche, Basel, Switzerland), digested with the appropriate restriction endonucleases (Roche) for 1 h at 37 °C, and cloned into plasmid pGEX-KG, previously digested with these enzymes. This was then transformed into *E. coli* XL1-Blue, and positive transformants were sequenced.

Table 1. Oligonucleotides for single spa domain constructs.
Restriction sites are indicated in boldface type.

Name	Sequence (5'-3')
Fw_*spaE*	CCG**GAATTC**ATGCTGCGCAACACGATGAAG
Rv_*spaE*	CCG**CCATGG**TTATTTTGGTGCTTGAGAGTCA
Fw_*spaD*	CCG**GAATTC**AAGCTGATGCGCAACAAAATAAC
Fw_*spaA*	CCG**GAATTC**AAGCTGATAACAATTTCAACAAAG
Rv_*spaD/A*	CCG**CCATGG**TTATTTCGGTGCTTGAGATTCG
Fw_*spaB*	CCG**GAATTC**AAGCGGATAACAAATTCAACAAAG
Fw_*spaC*	CCG**GAATTC**AAGCTGACAACAAATTCAACAAAG
Rv_*spaB/C*	CCG**CCATGG**TTATTTTGGTGCTTGAGCATCAT

Site-directed mutagenesis of a domain of spa

Mutations were introduced into domain D of protein A using a PCR-based mutagenesis strategy. Briefly, overlapping oligonucleotides carrying the desired mutation were combined with standard flanking primers to yield two overlapping mutant products. These were combined and amplified using the flanking primers alone to yield the mutant fusion product. In some cases, mutations were introduced using the Quikchange® method, according to manufacturer's instructions (Stratagene). The following amino acid substitutions were constructed: F5A, Q9A, Q10A, F13A, Y14A, L17A, N21A, R27A, N28A, G29A, F30A, I31A, Q32A, S33A, K35A, D36A, D37A, Q40A and E47A. A variant of Spa domain D lacking three additional codons unique to this domain was also created by PCR as previously described, generating a 58-residue variant [13]. Oligonucleotides used to introduce mutations are listed in Table 2. Amplimers were cloned directionally into pGEX-KG and expressed as described above.

Induction and purification of recombinant proteins

For expression of recombinant Spa truncates, pGEX-KG constructs were purified from *E. coli* XL1-Blue using the Wizard® plasmid purification kit (Promega Corp., Madison, Wis., USA) and transformed into *E. coli* TOPP3. Overnight cultures were inoculated into fresh medium and grown to an OD_{600} of 0.5. Isopropyl b-D-thiogalactopyranoside (IPTG) was added to a concentration of 1.5 mM and the culture was grown for a further 3 h. Cells were harvested by centrifugation at 7,000 rpm for 10 min in a Sorvall GS-3 rotor. The pellet was resuspended in NaCl/P_i containing protease inhibitor (Roche), lysozyme (200 μg/mL) and DNase I (3 μg/mL). Cells were lysed by repeated passage through a French Pressure Cell. Cell debris was removed by centrifugation at 17,000 rpm for 30 min in a Sorvall SS-34 rotor and the supernatant was filtered through a 0.45 μm filter. Recombinant proteins expressed from pGEX-KG contained an N-terminal glutathione-S-transferase (GST) fusion of 26 kDa. The GST-fusion proteins were purified using a GSTrap™ column (Amersham, Uppsala, Sweden) and dialysed against NaCl/P_i. Recombinant five- and single-domain GST-fusion proteins had approximate Mws of 59 kDa and 32 kDa, respectively. Recombinant V_H3-Fab was produced in *E. coli* XL1-Blue from pComb3:JMSpA3-08 as described previously [28]. Murine V_H4 IgM was produced as described elsewhere [29]. Recombinant full-length (FL) VWF and truncates D'-D3, A1, and A3 were cloned and expressed as described previously, from baby hamster kidney cells, *Pichia pastoris* and *E. coli*, respectively [18,30,31]. Recombinant VWF A1

Table 2. Oligonucleotides used for spaD variants.
Reverse primers for all variants is the reverse complement of forward primer.
Restriction sites are indicated in boldface type and underlined bases indicate a changed codon.

Name	Forward Primer (5'-3')
Fw_*spaD*$_{58}$	CCG**GAATTC**AAGCTGATAATAACTTCAACAAAG
F5A	GCAACAAAATAACGCGAACAAAGATC
Q9A	CTTCAACAAAGATGCACAAAGCGCC
Q10A	CAACAAAGATCAAGCAAGCGCCTTC
F13A	CAAAGCGCCGCGTATGAAATC
Y14A	GCGCCTTCGCGGAAATCTTG
L17A	CTATGAAATCGCGAACATGCC
N21A	GAACATGCCTGCGTTAAACGAAG
R27A	GAAGCGCAAGCTAACGGCTTC
N28A	CCAACGTGCGGGCTTCATTC
G29A	GCAACGTAACGCGTTCATTCA
F30A	GTAACGGCGCGATTCAAAGTC
I31A	GTAACGGCTTCGCGCAAAGTC
Q32A	GGCTTCATTGCGAGTCTTAAAG
S33A	GCTTCATTCAAGCGCTTAAAGAC
K35A	GTCTTGCGGACGACCCAAG
D36A	GTCTTAAAGCGGACCCAAGCC
D37A	CTTAAAGACGCGCCAAGCC
Q40A	GACGACCCAAGCGCAAGCACTAACG
E47A	CGTTTTAGGTGCAGCTAAAAAATTAAACG

domain was also produced in *E. coli* M15 cells harbouring plasmid pREP4 (Qiagen) from plasmid pQE30-VWF-A1 as described previously [17,32]. All VWF truncates contained an N-terminal histidine tag.

Protein analysis and Western ligand blotting assays

Recombinant proteins were analyzed by SDS-PAGE using gels containing 4.5-12.5% acrylamide. Gels were stained using Coomassie™ Brilliant Blue (Amersham) or by silver staining [33]. In some cases, proteins were transferred electrophoretically to polyvinylidene difluoride (PVDF) Western blotting membranes (Roche) by the wet system (Bio-Rad, Hercules, CA, USA) in Tris-HCl (0.02 M), glycine (0.15 M), and methanol (20 % v/v). Membranes were incubated for 15 h at 4 °C in 5 % blocking reagent (Marvel milk powder). Membranes were washed and incubated with gentle agitation with VWF as previously described [15]. F(ab')$_2$ were isolated from polyclonal anti-VWF-HRP (Dako, Glostrup, Denmark) by pepsin digestion (Pierce, Rockford, IL, USA) for detection of FL-VWF. VWF truncates were detected using HisProbe-HRP (Pierce). Membranes were developed using LumiGLO chemiluminescent substrate (New England Biolabs, Ipswich, MA, USA) according to the manufacturer's instructions.

Preparation of flow chamber slides

Purified VWF was used at a concentration of 100 μg/ml. A 500ml solution was applied to glass slides (75 x 25 mm) and allowed to attach for 2hrs at room temperature in a humidity chamber. Slides were washed 3 times in NaCl/P_i buffer to remove any unbound protein. Finally, the slides were blocked with 1% BSA for a further 1 hour at 37ºC.

Videomicoscopy

Overnight cultures of bacteria were washed twice in NaCl/P_i and resuspended to an OD_{600} of 1. Next, cells were perfused over immobilized VWF at various shear rates. A syringe pump (Harvard Biosciences, MA, USA) was used to aspirate bacteria through the flow chamber. Bacterial adhesion was visualized using phase contrast microscopy (63X LD-Achroplan objective) through the flow chamber (GlycoTech, Rockville, MD, USA) mounted on a Zeiss Axiovert-200 epi-fluorescence microscope (Carl Zeiss, Welwyn Garden City, UK). Images were captured every second up to 300s by a liquid chilled Quantix-57 CCD camera (Photometrics Ltd, Tuscon, AZ, USA). Bacterial adhesion was analysed using MetaMorph (Universal Imaging Corp., Downingtown, PA, USA).

Recombinant protein binding assay

Microtitre plates (Sarstedt, Nümbrecht, Germany) were coated with Spa or VWF in carbonate buffer overnight at 4 °C. Wells were washed three times with Tween 20 (0.05 % v/v) in PBS and blocked at 37 °C with 3 % (w/v) bovine serum albumin (BSA) in PBS for 2 h at 37 °C. Wells were again washed and varying concentrations of appropriate ligand in 3 % BSA were added. Plates were incubated for 1 h at 37°C. Unbound protein was removed by washing. Bound VWF was detected as before. Bound Spa was detected by HRP-chicken anti-GST antibody (Gallus Immunotech, Fergus, ON, Canada). After washing, 100 μl of a chromogenic substrate solution (1 mg/ml tetramethylbenzidine and 0.006 % H_2O_2 in 0.05 M phosphate citrate buffer pH 5.0) was added, and plates were developed for 10 min. The reaction was stopped by the addition of 2 M H_2SO_4 (50 μl/well), and plates were read at 450 nm. Data from ELISA-type assays was graphed and analysed using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego, California, USA.

Inhibition studies

ELISA plates were coated and blocked as before. Wells were incubated with mixtures containing increasing concentrations of inhibitor and a standard concentration of ligand corresponding to its half-maximal binding to the coated protein as determined by ELISA-type binding assays. Inhibition was performed using Spa and VWF truncates, and also the Fc portion of human IgG (Jackson Labs, Inc., Cambridgeshire, U.K.) and a V_H3-Fab fragment, produced in *E. coli* from plasmid pComb3::JMSpA3-08 as previously described [28]. Wells were washed and bound protein was detected as before. Percentage inhibition was calculated from the percentage of bound protein detected in the absence of inhibitor.

Results

Perfusion studies

To determine whether expression of protein A on the cell surface is sufficient for adhesion of bacteria to immobilized VWF under flow, *L. lactis* or *L. lactis* Spa$^+$ were perfused over glass slides coated with recombinant human VWF. Cells were perfused for 300 seconds, and adherent bacteria visualized by video microscopy. Adherent bacteria were observed only in the case of the *L. lactis* expressing protein A. Binding occurred at low shear rates (50 s^{-1}) but not at high shear rates (Figure 1A). Quantitative analysis was performed by counting adherent cells from at least three separate fields at 60 second time intervals (Figure 1B). This supports previous work suggesting that Spa on the surface of *S. aureus* is necessary for efficient attachment of bacteria to a VWF-collagen complex at low shear rates [26] by demonstrating that it is sufficient for this process.

Immobilised *L. lactis* or *L. lactis* Spa$^+$ were tested for their ability to recruit platelets in whole blood under various conditions of shear. None of the shear rates tested (50 s^{-1} to 1500 s^{-1}) supported platelet adhesion by *L. lactis* or *L. lactis* Spa$^+$ (data not shown).

Identification of regions within von Willebrand factor that bind to protein A

It was shown previously that VWF purified from human plasma bound to full length recombinant protein A with an apparent K_d of 15 nM measured by surface plasmon resonance [15]. Here ELISA-type binding assays were employed to study the interaction between recombinant protein A and recombinant VWF. Firstly, full-length (FL) recombinant VWF and the extracellular repeat region of Spa fused to GST (GST-SpaEDABC) were used. When FL-VWF was immobilized onto ELISA plates, binding of soluble GST-SpaEDABC occurred in a dose-dependent and saturable fashion with half-maximal binding at approximately 30 nM (Figure 2A). Similar data were obtained when soluble FL-VWF was tested in binding experiments with immobilized GST-SpaEDABC (Figure 2B). This showed that the Spa bound via its N-terminal EDABC domains to VWF specifically and with high affinity, and that recombinant human VWF behaved in a similar fashion to plasma-derived protein used previously.

In order to identify the binding site(s) for Spa in VWF, the D'-D3, A1 and A3 truncates of VWF were tested for their ability to bind to immobilized GST-SpaEDABC. The A1 domain of VWF bound to GST-SpaEDABC dose-dependently and saturably with half-maximal binding at 100 nm. Binding was also detected for the VWF D'-D3 domain but with a lower affinity (half-maximal binding at 250 nm). In contrast, the A3 domain of VWF did not bind to GST-SpaEDABC (Figure 3A). This shows that VWF contains a high affinity Spa binding domain in domain A1 and a lower affinity site in domain D'-D3. To demonstrate specificity of these interactions, VWF A1 and D'-D3 were tested for binding to immobilized GST-SpaEDABC in the presence of increasing concentrations of soluble GST-SpaEDABC. The soluble Spa inhibited binding in a dose-dependent and saturable manner, providing further evidence of the specificity of the interaction (Figure 3B).

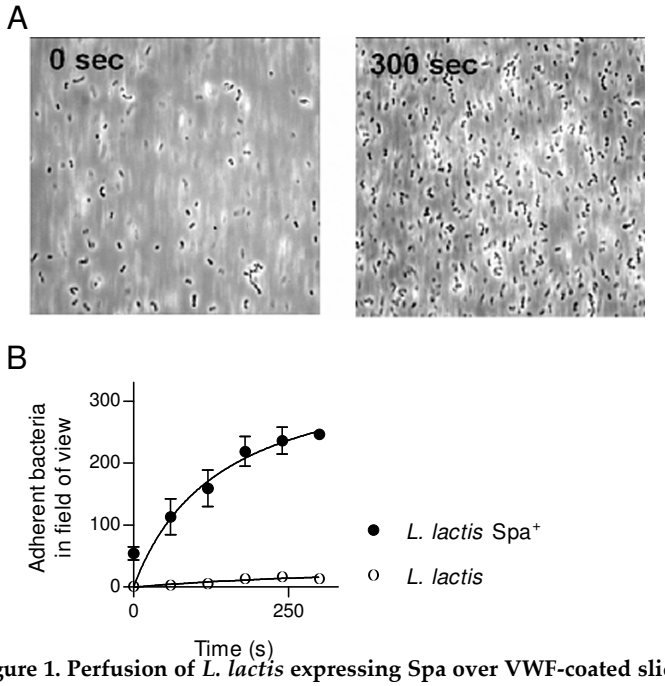

Figure 1. Perfusion of *L. lactis* expressing Spa over VWF-coated slides.
L. lactis or *L. lactis* expressing Spa were perfused over glass slides coated with full-length VWF (100 mg/ml). Live imaging of adherent cells by videomicroscopy was performed as described in "Experimental Procedures". **A**: images of *L. lactis* Spa+ after 0 and 300 seconds of perfusion over VWF. **B**: Adherent bacterial cells were counted from three independent fields of view at 60 second intervals for both *L. lactis* and *L. lactis* Spa+.

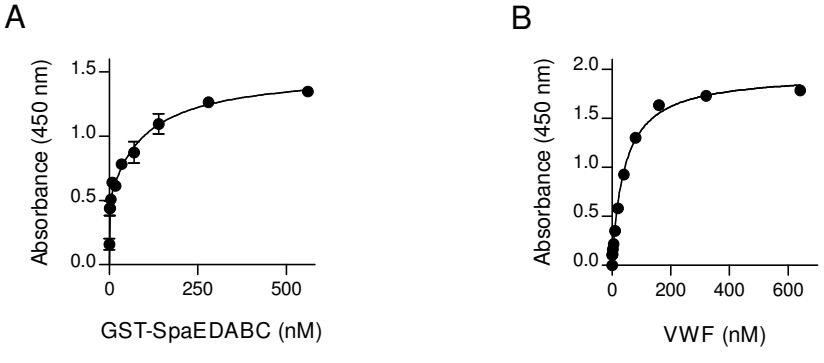

Figure 2. Interaction of full-length (FL-) VWF to GST-SpaEDABC.
A: Microtitre wells were coated with VWF (10 µg/mL) and incubated with increasing concentrations of GST-SpaEDABC. HRP-conjugated chicken anti-GST antibody was used to detect bound GST-SpaEDABC. In the reverse assay, binding of soluble VWF to immobilised SpaEDABC (10 µg/mL) was detected using HRP-anti-6xHis monoclonal antibody (**B**). Values are the means ± standard deviation of triplicate wells. The experiment was performed three times in triplicate with similar results.

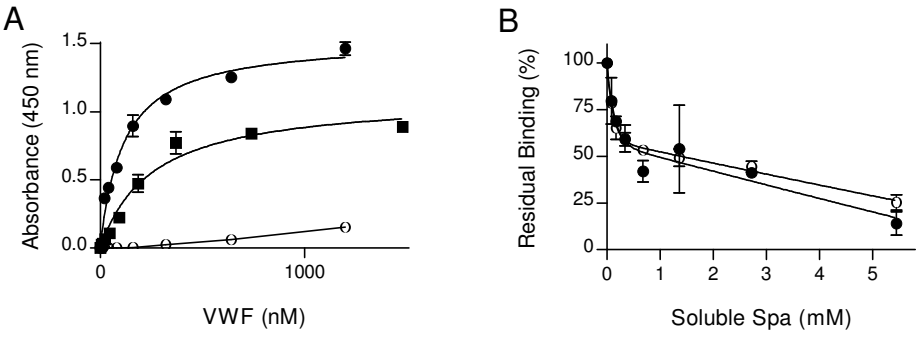

Figure 3. Binding of GST-SpaEDABC to recombinant VWF truncates.
A: Microtitre plates were coated overnight with GST-SpaEDABC (10 µg/mL) and incubated with increasing concentrations of VWF-D'D3 (squares), VWF-A1 (closed circles) or VWF-A3 (open circles). Bound vWF constructs were detected with HRP-anti-His monoclonal antibody. Half-maximal binding for VWF-A1 and VWF-D'D3 truncates was observed at 100 nm and 250 nm, respectively. **B:** VWF-A1 (closed circles, 100 nm) or VWF-D'D3 (open circles, 250 nm) were tested for binding to immobilised Spa in the presence of increasing concentrations of soluble Spa. Percentage binding relative to VWF binding in the absence of soluble Spa was calculated. Values are the means ± standard deviation of three separate experiments.

Individual domains of protein A bind von Willebrand factor

Each of the homologous domains E, D, A, B and C of protein A can bind individually to the Fc region of IgG and to the Fab region of IgM that of the V_H3 subclass [11,34]. In order to investigate if each domain can bind to VWF, GST-SpaE, GST-SpaD, GST-SpaA, GST-SpaB and GST-SpaC fusions were tested for their ability to bind to the VWF A1 domain by Western ligand blotting. When 250 ng of GST-Spa constructs were probed with 250 nm VWF A1, binding was detected for each of the Spa constructs except for GST-SpaE (Figure 4A). Weak binding was detected only when larger amounts of GST-SpaE were tested (Figure 4B).

Binding of Spa domains to VWF was also investigated with ELISA-type binding assays. Each GST-Spa truncate was immobilized and tested for its ability to bind soluble VWF A1 and D'-D3 proteins. The GST-Spa fusions bound to VWF A1 dose-dependently and saturably with similar affinity (half-maximal binding at approximately 100 nm) (Figure 4C). Each GST-Spa fusion also bound to the D'-D3 domain with similar affinities (not shown). It is noteworthy that GST-SpaE behaved in a similar fashion to the other GST-Spa constructs. The apparent lower affinity of the E domain for VWF A1 in ligand blotting could be explained by slower or improper renaturation of the protein after transfer from the SDS-PAGE gel to the PVDF membrane. Alternatively, reduced binding may be due to the detrimental effect of blotting procedure on folding of the SpaE domain. It is also worth noting that the E domain of Spa has the greatest amino acid sequence differences from the other Spa domains.

SpaD binding to VWF A1 is inhibited by IgG Fc regions but not a VH3-Fab

Hartleib *et al*. reported inhibition of the Spa-VWF interaction by IgG [15]. This suggests a possible shared binding region on Spa for IgG Fc and VWF. However, the large IgG molecule might have sterically blocked VWF binding. In addition, the pooled IgG used may have

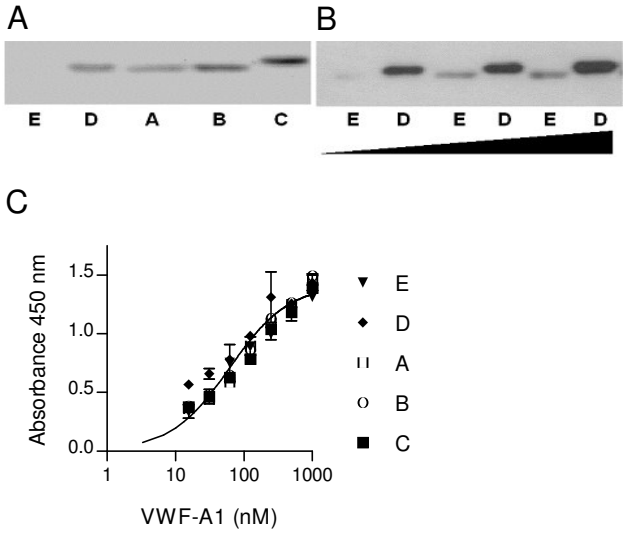

Figure 4. Interaction of single GST-Spa domains with VWF A1.
A: individual GST-Spa domains (250 ng) were separated by SDS-PAGE, immobilised on PVDF membrane and assayed for VWF-A1 binding by ligand affinity blotting. **B**: increasing concentrations of domains GST-SpaE and GST-SpaD (500 ng, 1mg, 2mg) were assayed by for VWF-A1 binding by ligand affinity blotting.
C: interaction of GST-SpaE, GST-SpaD, GST-SpaA, GST-SpaB and GST-SpaC domains to soluble VWF-A1 by ELISA-type assay. Values represent the mean of triplicate determinations. Binding curve represents average of all data values from GST-Spa truncates. Experiments were performed three times.

contained antibodies against Spa [15]. To overcome these potential problems, here the Fc fragment of IgG was used for binding and inhibition studies along with a recombinant V_H3 heavy chain fragment JMSpa3-08, with a non-reactive V_H4-bearing IgM included as a control. Binding of Spa to Fc and V_H3-derived immunoglobulin occurs through distinct binding regions within the Spa domain and is non-competitive, as demonstrated by the structures of complexes and by sandwich ELISA assays [12,13]. Inhibition studies performed here revealed that the Fc fragment blocked VWF A1 binding in a dose-dependent and saturable manner while the V_H3 heavy chain fragment had no effect (Figure 5) which strongly suggests that VWF A1 binds to the same region of Spa as the Fc fragment of IgG.

Amino acid substitutions of SpaD

GST-fusion proteins of Spa domain D bearing substitutions in residues known to be involved in Fc or V_H3-Fab binding [12,35] were generated, to identify the residues on Spa involved in binding to VWF A1. The D domain of Spa was chosen because its crystal structure has been solved. A 58-residue variant (GST-SpaD_{58}) of GST-SpaD, lacking the additional N-terminal residues unique to SpaD was also included in this study. A number of residues were substituted in order to investigate a role in VWF binding (Fig 6A). The substitutions generated were F5A, Q9A, Q10A, F13A, Y14A, L17A, N28A, I31A, K35A (Fc binding), G29A, F30A, S33A, D36A, D37A, Q40A E47A (V_H3-Fab binding), Q32A (proposed to have a minor

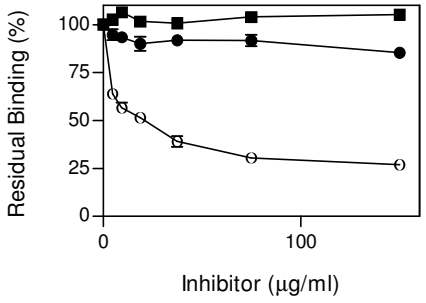

Figure 5. Inhibition of Spa binding to VWF-A1 by Fc and VH3-Fab.
96-well plates coated with GST-SpaD (10 µg/mL) were incubated with a mixture of VWF-A1 (100 nM) and various concentrations of Fcg(open cricles) or VH3-Fab (closed cricles). A VH4-bearing IgM (squares), which does not bind Spa, was also included. Bound VWF was monitored using a HRP-conjugated anti-Hisx6 antibody. Percentage inhibition was calculated relative to VWF bound in the absence of inhibitor. The experiment was performed three times with similar results.

```
                ←HELIX 1→      ←HELIX 2 →     ←  HELIX 3  →
    1     3       10         20         30         40         50       58
    |     |       |          |          |          |          |        |
D   ADAQQNNFNKDQQSAFYEILNMPNLNEAQRNGFIQSLKDDPSQSTNVLGEAKKLNESQAPK
E   AA----QHDEAQQNAFYQVLNMPNLNADQRNGFIQSLKDDPSQSANVLGEAQKLNDSQAPK
A   AD---NNFNKEQQNAFYEILNMPNLNEEQRNGFIQSLKDDPSQSANLLSEAKKLNESQAPK
B   AD---NNFNKEQQNAFYEILHLPNLNEEQRNGFIQSLKDDPSQSANLLAEAKKLNDAQAPK
C   AD---NKFNKEQQNAFYEILHLPNLTEEQRNGFIQSLKDDPSVSKEILAEAKKLNDAQAPK
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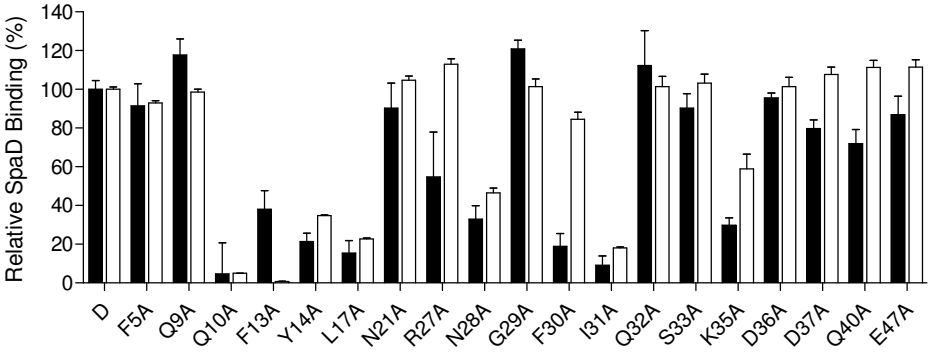

Figure 6. Effect on ligand binding of SpaD substitutions.
A: alignment of the five domains of protein A based on SpaD. Conserved residues are denoted by an asterisk (*), conserved substitutions in residues by a colon (:) and semi-conserved residues by a stop (.). Residues substituted or deleted by site-directed mutagenesis are highlighted grey. **B**: ELISA plates were coated with GST-SpaD variants (10 µg/ml) followed by incubation with rabbit IgG or VWF-A1 at concentrations corresponding to half-maximal binding to wild-type GST-SpaD as previously determined by ELISA. Adherent protein was detected as described in "Experimental Procedures". Experiments were performed in triplicate on three separate occasions. Figures represent the mean ± SD from three independent experiments.

contact in both interactions), N21A and R27A (conserved residues in all Spa repeats but not involved in Fc or V_H3-Fab binding). Variants were tested for binding to IgG and VWF A1 by ELISA-type assay. GST-$SpaD_{58}$ bound with a similar affinity to wild-type GST-SpaD to both ligands (not shown). The substitution of N21, which is not involved in Fc or V_H3-Fab binding also showed no reduction in VWF binding. Residues Q10, F13, Y14, L17, R27, N28, F30, I31 and K35 were shown to be important in VWF binding (Figure 6B). When these residues were mapped on the solved structure of SpaD, they were seen to form a cluster between helices I and II. With the exceptions of R27 and F30, these residues coincide with those involved in IgG Fc binding (Figure 6B) [35]. Interestingly, a similar binding profile was observed for GST-SpaD variants binding to the A1 and D'D3 domains (data not shown). In contrast, residues known to be involved in the V_H3-Fab binding (G29, S33 and D36, D37, Q40, E47) did not demonstrate reduced VWF binding when substituted.

Discussion

Protein A is one of a number of surface proteins expressed on the surface of *S. aureus* that is known to interact with plasma proteins. Several studies have demonstrated a role for protein A in staphylococcal virulence. Protein A-defective (Spa^-) mutants have reduced virulence in murine models of septic arthritis, septicaemia and skin abscesses, most likely due to the anti-phagocytic effect of Spa binding IgG Fc [36,37]. In a murine pneumonia model, Spa^- mutants showed reduced virulence where the pro-inflammatory properties of Spa binding to TNFR-1 were strongly implicated [38]. Spa has been shown to trigger apoptosis of murine B cells that express antigen-receptors with V_H3-Fab analogues, with greatest efficiency for marginal zone B cells that are involved in defense from blood borne infections. This likely contributes to immunosuppression during *S. aureus* infection [39].

Fluid-shear experiments in solution have suggested a function for Spa in promoting bacterial adherence to platelets in whole blood under very high shear rates (5000 s^{-1}) [25], such as those found in stenotic vessels [23]. However, under physiologically relevant shear conditions we could not detect capture of platelets by immobilized bacteria. Spa can mediate adherence of staphylococci to immobilized collagen under flow in the presence of VWF [26]. Also it has been shown that Spa can mediate adherence of *S. aureus* to VWF-coated surfaces in conditions of low shear [15]. Here, by expression of protein A on the surface of a surrogate bacterial host *L. lactis* at a level that is comparable to that of *S. aureus* strain Newman [6] in the absence of other surface proteins of *S .aureus*, we demonstrated that Spa is sufficient to support bacterial adherence to immobilized VWF under low shear conditions (50 s^{-1}), representing normal venous shear rates [23]. This is in agreement with previous studies using *S. aureus* cells that indicated that Spa was necessary for efficient binding [15,26]. Perfusions of whole blood at low shear rates across immobilized *S. aureus* leads to platelet capture and thrombus formation that was shown to be triggered by clumping factor A (Kerrigan *et al*., unpublished data). To determine if protein A contributed to this process, immobilized *L. lactis* expressing Spa was

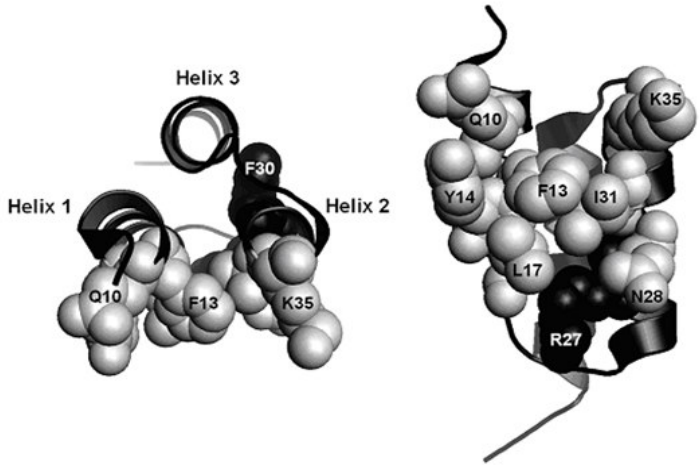

Figure 7. The Binding site for VWF-A1 on SpaD.
Ribbon diagram of SpaD from above (A) and the side (B) based its solved structure (PDB ID: 1DEE), with residues shown to be important in VWF-A1 binding highlighted in spacefill. Amino acids which also show an importance in binding IgG Fc are coloured grey, while those residues only affecting the VWF-A1 interaction are coloured black. The binding site for both VWF-A1 and IgG Fc is confined to the helix1-2 interface. Variant F30A shows a decrease in VWF-A1 binding, but is known to be important in the structural integrity of the Spa three-helix bundle.

investigated for its ability to recruit platelets. However, under all conditions of shear tested, no platelet adherence was observed, suggesting protein A is not involved in thrombus formation by *S. aureus*. It is more likely that Spa promotes bacterial binding to immobilized VWF, either bound directly to exposed subendothelial tissue or to platelets that had been previously captured. This opens the possibility that VWF contributes to the recruitment of Spa-expressing bacteria into VWF-rich platelet thrombi.

This study identifies two binding regions on VWF, domains, D'-D3 and A1, with half-maximal binding values of 250 nM and 100nM, respectively (Figure 3B). Specificity of the interactions is demonstrated by the ability of soluble Protein A to compete for binding to immobilized Spa (Figure 3B). The D'-D3 region of VWF is involved in binding and stabilizing blood coagulation Factor VIII. The A1 domain contains the binding site for the platelet receptor, GpIb. Spa binds to the D'-D3 and A1 domains with estimated half-maximal binding values of 250 and 100 nM, respectively. VWF has estimated dissociation constants for coagulation factor VIII, which it binds through the D'-D3 region, ranging from 200-400 pM [40,41]. However, it is reported that only one VWF subunit in fifty binds Factor VIII [41] . Therefore, it is possible that D'-D3 provides a more available, lower affinity site for Spa on VWF. The availability of binding sites on VWF is probably also limited by its globular shape [42,43] . The VWF A3 domain did not bind Spa (Figure 3A).

Each individual Protein A Ig-binding domain was tested for binding to VWF A1 by ligand affinity blotting. Binding was initially detected for domains D, A, B and C, but not domain E (Figure 4A). While there is high homology between Spa domains, there are some

differences (Figure 6A). Indeed, SpaE is the most heterologous Spa domain. An interaction was observed when increased amounts of Spa E were used (Figure 4B). When single Spa domains were tested for binding to VWF A1 by solid phase binding, all individual domains bound with equal affinity (Figure 4C), suggesting the reduced VWF binding observed may be due to the reported lower stability of domain E under denaturation-refolding conditions [44].

To help identify the region on Spa domain D responsible for VWF binding, the Fc region of human IgG and recombinant V_H3 heavy chain fragment JMSpa3-08 were tested for their ability to inhibit binding of Spa-D to VWF. The Fc fragment, but not the V_H3-Fab inhibited VWF A1 binding to Spa in a dose-dependent and saturable manner (Figure 5). This suggested the binding site for VWF on Spa domain D lies in a similar region to that of the Fc binding site. Site-directed mutagenesis of residues on Spa domain D confirmed that helices I and II contain the VWF binding site as well as that of Fc, with residues Q10, F13, Y14, L17, N28, I13 and K35 important for interactions with both ligands. This is likely due to specific contacts as previous mutational studies in this region indicate that Spa maintains its native structure [45-47]. However, the binding site is not identical as the substitution of R27A reduced binding to VWF A1, but not IgG (Figure 6B). This residue lies in helix II of Spa, and its side-chain points between helices I and II, consistent with the inhibition studies (Figure 7). With the exception of F30, substitution of residues that are involved in binding V_H3-Fab had no effect on binding to VWF A1 (Figure 6B). This is consistent with the inability of recombinant V_H3-Fab to inhibit binding of Spa to VWF A1 (Figure 5). The observed reduction in binding of the F30A variant may be due to structural changes in the Spa protein because the side chain of F30 contributes to the hydrophobic core of the protein A helical structure, lying between L44 and L51 of helix III (see Figure 6A, 7). Indeed, an F30A substitution in Spa has previously been shown to have dramatically decreased structural stability, despite still maintaining its affinity for the Fc region of IgG [46-48]. This is not the case for VWF A1. This may also explain the minor reduction in binding of variants D37A, Q40A and E47A, as helix III is involved stabilizing the three-helical structure of Spa [49]. Relative binding of the Spa substitutions to IgG (Figure 6B) were in agreement with previous reports [35,48].

In conclusion, we have demonstrated a direct role for Spa in adherence to surfaces through its interaction with VWF under flow. Mutagenesis studies on Spa have defined the binding site on Spa for VWF as lying between helices I and II, the same region that is responsible for the Fc interaction. All five Spa domains are capable of binding VWF, which may reduce competition for binding sites by Fc and VWF. Full-length Spa has a binding stoichiometry of two IgG molecules per molecule of Spa [50,51]. Indeed, VWF interactions occur in the presence of normal serum levels of IgG [15]. This region of Spa could be a potential therapeutic target; however, further studies to define the specific role *in vivo* of the interaction are required.

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CHAPTER 8

CHAPTER 8

General discussion

VWF plasma levels

Von Willebrand factor (VWF) plays an essential role in the haemostatic process and therefore circulating levels of VWF need regulation [1,2]. This is illustrated by the association between decreased VWF plasma levels and an increased bleeding tendency on one hand [3], and elevated VWF levels that predispose to cardiovascular mortality on the other [4,5]. In the studies presented in this thesis, we investigated various aspects that influence VWF plasma levels. These studies could provide new insights in the mechanisms responsible for the clearance of VWF from the circulation. Manipulation of these mechanisms may improve VWF half-life and as a consequence factor VIII (FVIII) half-life, which can be beneficial for the treatment of patients suffering from von Willebrand disease (VWD) or haemophilia A.

Glycosylation of VWF influences its plasma levels

Glycosylation contributes to correct folding, intracellular routing and/or optimal functionality of proteins [6-8]. Moreover, changes in the glycosylation profile could affect the survival of proteins in the circulation [9]. As outlined in chapter 2, we focussed on O-linked carbohydrates of VWF, which mainly consist of the sialylated T-antigen (NeuAc(α2-3)Gal(β1-3)(NeuAc(α2-6))GalNAc, figure 2, introduction) [10]. We found an inverse relation between the extent of sialylated T-antigen on VWF and VWF plasma levels among normal individuals (figure 2, chapter 2), patients suffering from liver cirrhosis (figure 3, chapter 2) and VWD type 1 patients (figure 4, chapter 2). Moreover, we observed a positive correlation between propeptide/VWF ratio and levels of sialylated T-antigen on VWF in VWD type 1 patients (figure 5, chapter 2), suggesting that sialylated T-antigen structures on VWF affect its clearance [11].

Genetic diseases caused by defects in the synthesis of the carbohydrate moiety of glycoproteins or in the attachment of glycans to these proteins are a known phenomenon [12-14]. Mutations in diseases like Walker-Warburg syndrome, muscle-eye-brain disease and congenital muscular dystrophy all cause underglycosylation of the protein dystroglycan with O-linked carbohydrates [14,15]. Furthermore, a number of diseases are associated with altered O-linked glycosylation. T-synthase (β1-3galactosyltransferase) is the enzyme capable of adding galactose to N-acetylgalactosamine. This results in the T-antigen, a carbohydrate that is part of the O-linked carbohydrates on VWF (figure 2, introduction) [16]. Deficiency of T-synthase activity is associated with to a number of diseases, such as IgA nephropathy, Henoch-Schönlein purpura and Tn syndrome [14,17,18]. The Tn syndrome is associated with a mutation in *Cosmc*, a gene that encodes a molecular chaperone, that is required for proper folding and hence full activity of T-synthase [19-21]. Tn syndrome is associated with neutropenia, mild haemolytic anaemia and thrombocytopenia [22,21]. Sialoglycoproteins on platelets of patients with Tn syndrome do not express T-antigen on their surface. However, these platelets do agglutinate in the presence of ristocetin and also aggregation by ADP and collagen is normal [22]. VWF half-life of these patients might be affected and based on our data one would suspect the half-life to be increased, although these patients show bleeding. It would therefore be interesting to investigate the ability of VWF from these patients to bind its partners, for instance platelet-receptor GPIbα. Increased affinity towards GPIbα might explain the

thrombocytopenia that is observed in these patients.

We observed substantial differences in the level of sialylated T-antigen on VWF from different individuals (chapter 2). It is difficult to imagine that O-linked glycosylation sites contain more than one T-antigen per site, since there is only one attachment site per serine or threonine residue present at the protein backbone [16]. A possible explanation of altered sialylated T-antigen levels is an increased number O-linked glycosylation of serine and threonine residues. This would imply that VWF derived from patients with increased levels of sialylated T-antigen (e.g. patients suffering from VWD type 1) contains additional sialylated T-antigen structures when compared to patients with decreased levels of sialylated T-antigen (e.g. patients suffering from liver cirrhosis). Many O-linked glycans are completed without elongation or termination of the core structures, or with partial elongation. This apparently incomplete synthesis is a result of the speed or volume of throughput within the processing pathways of the Golgi apparatus leading to an insufficient supply of transferases or available sugarnucleotides [16]. This might explain the correlation between expressed decreased levels of T-antigen on VWF and increased VWF (chapter 2). Synthesis of these apparently 'incomplete' glycans has functional significance [16,23,24]. Lau *et a*l showed that branching of N-linked glycans cooperates with number of N-glycans on glycoproteins in response to the hexosamine flux, resulting in differences in levels of N-linked glycans and in carbohydrate-structure. They suggest that this results in cellular transition between cell growth and arrest/differentiation, due to different carbohydrate-composition of glycoproteins [24]. There might be a similar system for O-linked glycans, which would explain the differences in sialylated T-antigen levels expressed on VWF. This might suggest that VWF multimers with different carbohydrate content display different affinity for a number of proteins and have therefore different function and/or clearance rate.

Altered levels of sialylated T-antigen on VWF can also potentially be caused by mutations or polymorphisms in genes encoding enzymes responsible for its O-linked glycosylation. When mutations in genes encoding enzymes responsible for construction of sialylated T-antigen, affect the level of sialylated T-antigen on glycoproteins, the effect should be notable on more proteins than just VWF. However, tissue-specific alterations in glycosylation of the same protein have been described [16,25,26]. VWF multimers are produced in Weibel-Palade bodies in endothelial cells and a-granules in megakaryocytes. It would be of relevance to investigate whether plasma levels of other endothelial Weibel-Palade body derived glycoproteins than VWF also correlate with sialylated T-antigen levels on their surface. Propeptide and P-selectin are examples of these proteins, but they are not O-linked glycosylated and do therefore not contain T-antigen. Alternatively, mutations in the VWF gene might result in changes in N- and O-linked carbohydrate content of VWF. Mutations located near or at a glycosylation site could potentially alter the glycosylation profile of VWF multimers. Most carbohydrates on glycoproteins are covered by sialic acid residues, which are negatively charged. Changes in glycosylation profile of a protein can induce changes in charge at the protein-surface,which could interfere with the interaction between VWF and other proteins such as its clearance receptor. In addition, such mutations could hamper the

tertiary structure of VWF, resulting in intracellular retention in endothelial cells and/or immediate removal from circulation after secretion. It would be of importance to determine the glycosylation pattern of VWF from patients with different levels of sialylated T-antigen. Several groups have identified the glycan content of glycoproteins in patient populations using mass spectrometry [27-29]. This provides a useful tool to determine the number of sialylated T-antigen residues on VWF.

Cysteine mutations in VWF lead to altered VWF plasma levels

In order to obtain functional multimerized VWF, it is essential that the protein is correctly processed. Multimerization of VWF monomers is a delicate process that requires several separate steps. VWF multimers contain disulphide bonds that link monomers into multimers (Introduction, Figure 1). Disulphide bonds require cysteine residues and all cysteine residues in the mature VWF subunit participate in these bonds [30]. Several mutations identified in VWD patients replace a cysteine residue, thereby affecting disulphide bonding in VWF. Little is known concerning the relationship between cysteine mutations in VWF and its survival in the circulation. Chapter 3 describes the evaluation of the effect of three VWD-associated cysteine mutations on the survival of VWF. Two of the investigated mutations (VWF/C1130F and VWF/C1149R) are located in the VWF-D'D3 region and have been identified in patients with VWD type 1. The third mutation (VWF/C2671Y) was identified in a patient with VWD type 3 and is situated at the carboxyterminal region of VWF. In vitro analysis of the mutants revealed an impaired secretion for all three mutants [31]. However plasma levels of patients carrying the mutations were reduced to a larger extent as would be expected from VWF levels observed in in vitro experiments [32,33]. This might point to increased clearance of these mutants. Therefore, we examined VWF half-life in these patients by administration of DDAVP. Patients showed four to five fold decreased VWF half-life when compared to healthy controls. Increased clearance of these mutants was confirmed in an in vivo model using VWF deficient mice [34]. Increased removal from the circulation was observed for a number of other VWF variants as well, like VWF/S2179F (located in VWF-D4), VWF/W1144G (located in VWF-D3) and VWF/R1205H (Vicenza mutant, located in VWF-D3) [34,35]. It is of interest that the majority of these mutations are located in the VWF-D'-D3 region of VWF, suggesting that variations in this region might render VWF more or less susceptible to clearance.

The VWF variants described in chapter 3 contain cysteine mutations, which affect formation of disulphide bridges. They may therefore result in conformational changes in VWF multimers. As a consequence, the interaction with proteins binding to this VWF region (such as FVIII and propeptide) could be altered. We found that both VWF/C1130F and VWF/C1149R mutations were associated with impaired binding to FVIII and propeptide. A number of other cysteine-mutations have been reported that are associated with decreased FVIII binding as well as reduced VWF levels [37,38]. It is not unthinkable that these mutant VWF molecules are also subject to increased clearance.

Self-association of VWF

To prevent spontaneous binding of VWF to platelets, VWF circulates in a globular, inactive conformation. If VWF would circulate in the platelet-binding conformation, spontaneous binding of VWF to platelets could occur and subsequent obstruction of the vascular system. The interaction between platelets and VWF is mediated by binding of platelet receptor GPIbα to VWF-A1. One of the differences between the inactive and platelet-binding conformation is that the GPIbα-binding site within VWF-A1 is cryptic in the inactive conformation. It has been proposed that domains flanking VWF-A1 (VWF-D'D3 and VWF-A2 and VWF-A3) cover VWF-A1 in its inactive conformation [39-41]. However, it has not been elucidated which VWF domains interact with each other in order to maintain this conformation. Transformation of VWF to its platelet-binding state involves several stages [39]. It would therefore be interesting to investigate the contribution of VWF-D'D3 to this proces. It is possible that intermolecular disulphide bridging is needed for optimal self-association between VWF-D'D3 and VWF-A1 or with one of its flanking domains. As described above, mutations affecting disulphide bridges display impaired binding to FVIII and propeptide [37,38], whereas binding to the potential clearance receptors is apparently enhanced (chapter 3) [42]. This might suggest that impaired disulfide bridging in VWF-D'D3 could also modulate the association between VWF-D'D3 and VWF-A1, VWF-A2 or VWF-A3.

The interaction between VWF-D'D3 and VWF-A1, VWF-A2 or VWF-A3 might also be facilitated by carbohydrates present on the N-terminal part of the VWF molecule. VWF-D'D3 region contains 3 N-linked glycans, O-linked glycans on VWF are predominantly located flanking the VWF-A1, VWF-A2 contains 2 N-linked glycans and there is one O-linked glycan present in the area between VWF-A2 and VWF-A3 (figure 1, introduction). If glycans on VWF are involved in this interaction, they either may remain cryptic in the inactive state of VWF or be exposed at the surface of the VWF molecule. One could also think of mixed types of intermediates where both N-linked and O-linked glycans contribute to the interaction between the various domains. Interference in this interaction, by either disrupted disulphide bonding or altered glycosylation profile, might result in conformational changes in the VWF multimers. This might affect the interaction between VWF and other proteins such as its clearance receptor. We showed that the level of O-linked glycosylation on VWF is associated with VWF plasma levels (chapter 2) [43]. However, several reports show that VWF antigen levels are associated with blood group, indicating that the N-linked glycans on VWF influences its plasma levels [44-47]. This proposes that N-linked and O-linked glycans contribute to the clearance of VWF. This might suggest that VWF-D'D3 interacts with VWF-A1 or its flanking domains, leaving both N-linked and O-linked glycans available at the surface of the protein, making sure that they are available for interaction with its clearance receptor.

Interaction with macrophages

When the molecular mechanisms responsible for the clearance of VWF are clarified, therapeutic strategies can be developed resulting in a recombinant VWF with increased half-life. Therefore the goal of the study described in chapter 5 was to elucidate possible mechanisms contributing to clearance of VWF. We describe the interaction of FVIII, VWF and the FVIII/VWF complex with macrophages present in spleen and liver.

A large number of glycoproteins are removed from the circulation in the liver via their carbohydrate moiety [16,48-53]. Liver cells express carbohydrate-specific receptors directed against a number of carbohydrate structures [9]. In chapter 5 the uptake of VWF by Kupffer cells in the liver is described. The results of the experiments described in chapter 5 could indicate that Kupffer cells are players in the removal of VWF from the circulation. However, no receptor responsible for the uptake of VWF has been identified yet. Since glycosylation profile of VWF influences its plasma levels, this suggests carbohydrates on VWF are the target of a possible clearance receptor for VWF on Kupffer cells. Several carbohydrate receptors have been identified on Kupffer cells. One of these receptors is the Kupffer cell receptor, that recognizes glycoproteins that carry N-acetylglucosamine and mannose terminated glycoproteins, however, this receptor is not expressed on human Kupffer cells [54,55]. Another receptor is a fucose receptor that binds to both fucose and galactose terminated glycoproteins [56,57]. Kupffer cells also express the mannose receptor, that recognizes mannose and fucose 58. In vivo experiments showed that both mannose and fucose labelled BSA are targeted to Kupffer cells in the liver [59]. Fucosylated BSA was taken up via mannose and fucose receptors on Kupffer cells, while uptake of mannosylated BSA by Kupffer cells is mediated only by mannose receptors [59]. Moreover, $GdCl_3$ pretreatment of rats completely inhibited the binding of both mannosylated and fucosylated BSA to Kupffer cells [60]. We also observed inhibition of clearance when VWF deficient mice were treated with $GdCl_3$ prior to VWF injection (figure 4, chapter 5). N-linked glycans on VWF contain galactose, mannose and fucose (figure 2, introduction). One of the carbohydrate-specific receptors on Kupffer cells could be a possible candidate involved in removal of VWF from the circulation. The receptors mentioned above are directed against carbohydrate structures present on N-linked glycans. Our data show that O-linked glycosylation also affects VWF plasma levels (chapter 2). The O-linked glycans on VWF are predominantly composed of the sialylated T-antigen, which contains a terminating galactose and might therefore be a target for the fucose receptor on Kupffer cells. Moreover, Biessen and co-workers showed that recognition of fucose- and galactose-exposing proteins by the fucose receptor on Kupffer cells is strongly dependent on size. The K_i decreased with increasing ligand sizes and levelled of at 18 nm [61]. VWF multimers secreted in plasma consist of flexible strands varying in length up to 2 mm, that are composed of dimeric units (protomers) polymerized linearly in an end-to-end fashion through disulphide bonds [62]. Since VWF is one of the largest plasma proteins, it might be a suitable target for binding to fucose receptors. However, it needs to be investigated which receptor on Kupffer cells can interact with VWF. It might therefore be interesting to use proteomics or si-RNA as a tool to elucidate the clearance receptor for VWF on Kupffer cells.

Blood group determinants of non-O carriers terminate in fucose together with either galactose or N-acetylgalactosamine. Determinants from blood group O carriers terminate in fucose (Introduction, figure 2). Blood group O individuals display decreased VWF levels, which can be explained by shorter VWF survival rates [44-47,53,63]. The secretor locus affects α(1,2)fucosyltransferase, which adds terminal fucose to glycans [64]. However, there is conflicting evidence whether individuals with *Se*/*Se* show altered VWF plasma levels [65,66]. These observations might suggest that clearance in individuals is determined by blood group determinants and blood group O determinants are a better target for the clearance receptor when compared to non-O carriers. However, individuals with the Bombay phenotype, who totally lack H-antigen on VWF have even lower VWF levels than blood group O carriers [67]. This indicates that not only blood group determinants contribute to the clearance of VWF.

Role of VWF in development of FVIII inhibitors

One of the current problems, encountered in the treatment of Haemophilia A is the development of inhibitory antibodies against infused FVIII. The incidence of inhibitors is influenced by various patient-related factors such as mutation type or severity of the disease [68]. It is a current issue of debate whether preparations containing VWF are associated with a reduced risk of inhibitor development in these patients [69-73]. However, in vivo mouse experiments seem to be in favour of a protective effect by VWF, although this may depend on the experimental strategy employed [74,75]. Also in vitro experiments point to a protective effect of VWF: VWF interferes with uptake of FVIII by dendritic cells, thereby inhibiting the subsequent FVIII-induced T-cell response [76-78]. Like dendritic cells, macrophages can serve as antigen-presenting cells in order to cause antigen-specific T-cell responses. As described in chapter 5, we observed that the FVIII/VWF complex is targeted to macrophages within the spleen. Spleen macrophages that are located in the marginal zone have been reported to play an important role in the development of antigen-specific immune-tolerance [79] . The uptake of the FVIII/VWF complex by these cells might contribute to such tolerance. If so, there should be a difference after injection of FVIII alone or of the FVIII/VWF complex. In literature, it has been suggested that plasma-derived FVIII/VWF preparations might contain small amounts of TGF-β. TGF-β is a cytokine known to contribute to the development of regulatory T-cells that suppress antigen-induced T-cell responses. Another option is that once taken up by macrophages, the VWF/FVIII complex and FVIII alone follow distinct intracellular pathways, in which FVIII alone is presented to the MHC-II system more efficiently. Of interest, it has recently been reported that increased susceptibility to lysosomal proteolysis is associated with a reduced immunogenicity [80]. In view of the rapid degradation of VWF by macrophages compared to the relative slow degradation of FVIII (chapter 5, [81]), it cannot be excluded that different intracellular routings may contribute to differences in immunogenicity between FVIII and the FVIII/VWF complex.

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Nederlandse samenvatting

Vorming van een stolsel

Een goede bloedsomloop is essentieel voor een goed functionerend lichaam. Ons bloedvatenstelsel is een gesloten systeem waarin het bloed stroomt. Als een bloedvat beschadigd raakt ontstaat er het gevaar voor bloedverlies. Zonder tegenreactie kan dit leiden tot ernstige bloedingsproblemen. Daarom moet bloedverlies zo snel mogelijk worden gestopt en moeten beschadigingen snel en efficiënt worden gerepareerd. Hierbij worden lokaal een groot aantal bloedplaatjes (een van de componenten in het bloed) ingeschakeld. De bloedplaatjes kleven aan de plaats waar de vaatwand beschadigd is en gaan samenklonteren. Hierdoor wordt een plaatjesprop gevormd die beschadiging repareerd. De gevormde plaatjesprop is echter broos, daarom wordt het systeem van bloedstolling in werking gesteld waarbij meer dan 10 eiwitten in een kettingreactie op elkaar inwerken. Dit leidt tot de vorming van fibrine. Fibrine stabiliseert de plaatjesprop wat resulteert in een stevig stolsel.

Von Willebrand factor

Het plasma-eiwit von Willebrand factor (VWF) speelt een belangrijke rol bij de vorming van het stolsel. VWF zorgt er namelijk voor dat bloedplaatjes uit de bloedcirculatie op de plaats van de vaatwandbeschadiging ingesloten worden. Hiervoor bindt VWF eerst aan structuren (voornamelijk collageen) die vrij komen te liggen in de vaatwand bij beschadiging van een bloedvat. Alleen wanneer VWF zich hieraan vastgehecht heeft, is het in staat om passerende bloedplaatjes uit de bloedstroom te binden. Deze bloedplaatjes zullen vervolgens geactiveerd worden en een bloedplaatjesprop vormen die het bloeden moet tegenhouden. Daarnaast vormt VWF een complex stollingsfactor VIII (FVIII), waardoor FVIII stabieler wordt. FVIII is een van de eiwitten van de eerdergenoemde kettingreactie die leidt tot fibrinevorming. Door een complex te vormen met VWF, wordt het beschermd tegen afbraak.

VWF moet in voldoende mate en functioneel aanwezig zijn om te kunnen binden aan de bloedplaatjes, collageen in de vaatwand en stollingsfactor VIII. Verhoogde concentraties VWF in het plasma zorgen voor een verhoogd risico op hart- en vaatziekten. Terwijl te lage VWF waarden resulteren in de ziekte van von Willebrand (VWD; Von Willebrands Disease). Deze ziekte is in 1926 ontdekt door de Finse wetenschapper Erik Adolf von Willebrand en is de meest voorkomende erfelijke bloedingsziekte bij de mens. VWD uit zich in tal van bloedingproblemen zoals neusbloedingen, overvloedig menstrueel bloedverlies en post-operatieve en maag-darm bloedingen. Patiënten met VWD worden onder andere behandeld met medicijnen die VWF bevatten, zodat de bloedingen gestopt kunnen worden.

De ernst van deze ziekte varieert sterk en is afhankelijk van het type defect. Patiënten met VWD kunnen worden onderverdeeld in twee groepen. De eerste groep bestaat uit patiënten met een kwantitatief defect in hun VWF (VWD type 2), deze patiënten maken VWF dat niet functioneel is. Patiënten met een kwalitatief defect in hun VWF (VWD type 1 en 3) hebben lage VWF concentraties in het bloed, waarbij type 1 correspondeert met een gedeeltelijke en type 3 met een bijna volledige afwezigheid van VWF in het plasma. Deze lage waarden kunnen worden verklaard door enerzijds een verlaagde productie van VWF of een versnelde klaring (verwijdering) van het eiwit uit het plasma.

Het onderzoek beschreven in dit proefschrift richt zich op aspekten die van invloed zijn op de hoeveelheid VWF in het bloed en op het mechnisme dat ervoor zorgt dat VWF uit het bloed verwijderd wordt. Daarnaast hebben we ook gekeken waar VWF bindt aan Protein A, een eiwit dat zich op de bacterie *Staphylococcus aureus* bevindt.

O-gekoppelde suikers op von Willebrand factor

VWF is het grootste eiwit dat in het bloed aanwezig is en heeft een zeer complexe structuur (figuur 1, hoofdstuk 1). Elk VWF molecuul bestaat uit 20 tot 50 gelijke eenheden die als schakels van een ketting met elkaar verbonden zijn. VWF is een glycoproteine wat betekent dat het eiwit bedekt is met suikerstructuren. Er zijn 2 groepen suikerstructuren te onderscheiden, de N-gekoppelde suikers, die vast zitten aan aspargine aminozuren (bouwstenen van een eiwit) in VWF en de O-gekoppelde suikers, die aan seronine of threonine aminozuren in VWF gekoppeld zijn. Eerder onderzoek heeft uitgewezen dat suikerstructuren, aanwezig op VWF invloed kunnen hebben op de VWF-concentratie in het bloed. Een voorbeeld hiervan is zijn de bloedgroepsuikers. Een bloedgroep wordt bepaald door een type suikerstructuur dat zich op eiwitten bevind. Mensen met bloedgroep O hebben over het algemeen lagere VWF concentraties dan mensen met een andere bloedgroep. Dit zou kunnen betekenen dat het suikerpatroon op VWF invloed heeft op de hoeveelheid VWF in het bloed. In hoofdstuk 2 van dit proefschrift hebben we ons daarom geconcentreerd op de O-gekoppelde suikers op VWF. We hebben een methode ontwikkeld om de hoeveelheid O-gekoppelde suikers, aanwezig op VWF te kunnen meten. Met behulp van deze methode hebben we laten zien dat gezonde personen verschillende hoeveelheden O-gekoppelde suikers op hun VWF hebben (figuur 2, hoofdstuk 2). Het VWF van mensen met hogere VWF concentraties in hun bloed, bevat minder O-gekoppelde suikers in vergelijking met mensen met lagere VWF waarden. Daarna hebben we gekeken of dit fenomeen ook zichtbaar was in patiënten met extreem hoge of juist lage VWF waarden. Ook hieruit kwam naar voren dat naarmate het plasma van de patiënten minder VWF bevatte, dit VWF juist meer O-gekoppelde suikers bevatte (figuren 3 en 4, hoofdstuk 2). Bijvoorbeeld, patiënten met VWD type 1 hebben gemiddeld ongeveer 2 keer zoveel O-gekoppelde suikers op hun VWF in vergelijking met gezonde individuen. Hierna hebben we gekeken naar de verhouding tussen VWF en het propeptide (een eiwit dat gelijk met VWF door cellen wordt uitgescheiden) in de VWD type 1 patiënten. De verhouding tussen de concentratie van deze twee eiwitten in het bloed geeft namelijk informatie over de snelheid waarmee VWF uit het bloed verwijderd (geklaard) wordt. We vonden dat deze verhouding een relatie had met de hoeveelheid O-gekoppelde suikers op VWF (figuur 5 , hoofdstuk 2). Blijkbaar heeft de hoeveelheid O-gekoppelde suikers, aanwezig op VWF, invloed op de snelheid waarmee VWF uit de circulatie wordt geklaard (door een nog onbekend mechanisme).

Cysteine mutaties von Willebrand factor in patiënten met von Willebrands ziekte

VWF bevat een groot aantal van deze cysteines (een van de 20 aminozuren waaruit eiwitten zijn opgebouwd). Cysteines zijn van groot belang voor de structuur en vorm van een eiwit, omdat ze zwavelbruggen met elkaar kunnen vormen. Als een van deze cysteines wegvalt is het denkbaar dat dit een groot effect heeft op de vorm van het eiwit.

Patiënten met de ziekte van von Willebrand (VWD) hebben een mutatie in een gen (vaak het gen voor VWF) dat ervoor zorgt dat VWF van deze patiënten niet goed werkzaam is. Veel van deze mutaties zijn bekend en we hebben voor drie van deze mutaties gekeken of zij invloed hebben op de overlevingstijd van VWF (hoofdstuk 3). Deze drie mutaties resulteerden allemaal in de verandering van een cysteine aminozuur in het eiwit. We vonden dat de gemuteerde varianten sneller werden verwijderd uit de circulatie, in vergelijking met het normale VWF. We hebben de functionaliteit van de drie gemuteerde VWF eiwitten met verschillende testen onderzocht. Geen van deze testen kon verklaren waarom de gemuteerde eiwitten sneller uit het plasma werden verwijderd dan normaal VWF. Het zou mogelijk kunnen zijn de mutaties de vorm van het eiwit veranderen en dat het eiwit sneller uit het bloed verwijdert wordt, omdat het er anders uit ziet.

Halfwaardetijd van FVIII en von Willebrand factor

In het plasma circuleert 95-98% van het totale aanwezige FVIII in complex met VWF, terwijl maar 2-5% van het totale VWF gebonden is aan FVIII (figuur 1, hoofdstuk 6). Dit betekent dat bijna al het FVIII gebonden aan VWF circuleert, terwijl maar heel weinig VWF aan FVIII gebonden is in het bloed. De vorming van het complex stabiliseert FVIII en zorgt ervoor dat FVIII langer in de circulatie aanwezig blijft. De FVIII concentratie in het plasma is dan ook afhankelijk van de VWF concentratie. Dit word geïllustreerd in patiënten met de kwantitatieve vorm van VWD, zij hebben naast lage VWF waarden ook verlaagde FVIII waarden. Een tekort aan werkzaam FVIII veroorzaakt hemofilie A.

Omdat de concentratie VWF in het plasma invloed heeft op de hoeveelheid FVIII, zouden de halfwaardetijden (tijd die nodig is om de concentratie van een eiwit te halveren) van beide eiwitten een relatie met elkaar moeten hebben. Dan zou, als de halfwaardetijd van VWF bekend is, die van FVIII kunnen voorspeld kunnen worden. Om hemofilie A patiënten zo goed mogelijk te behandelen is het nodig de FVIII half waarde tijd te bepalen. Het zou makkelijk zijn als deze voorspeld zou kunnen worden. In een groep patiënten met ernstige hemofilie A hebben we de halfwaardetijd van FVIII en VWF bepaald. Zoals verwacht vonden we een relatie tussen de halfwaardetijden van deze twee eiwitten (figuur 1, hoofdstuk 4). Omdat de bloedgroep invloed heeft op VWF concentraties in het bloed, hebben we gekeken of er verschillen waren in de relatie tussen hemofilie A patiënten met een verschillende bloedgroep. Het was verrassend dat bij de patiënten met bloed groep O de relatie tussen de beide half waarde tijden beter is dan bij patiënten die geen bloed groep O hebben. Om de FVIII halfwaardetijd voor ieder persoon goed te kunnen voorspellen zijn er formules ontworpen in hoofdstuk 4 voor personen met een verschillende bloedgroep.

FVIII en Von Willebrand factor binden aan macrofagen

Omdat er een relatie bestaat tussen de halfwaardetijden van FVIII en VWF, zou het zo kunnen zijn dat deze eiwitten ook gezamenlijk uit de circulatie verwijderd worden. Over het mechanisme dat verantwoordelijk is voor de klaring van VWF is nog weinig bekend. Meer informatie over dit mechanisme zou kunnen leiden tot verbeterde behandeling van patiënten met VWD of hemofilie A. Daarom zijn we op zoek gegaan naar de cellen die verantwoordelijk zijn voor het verwijderen van FVIII en VWF uit het lichaam. In hoofdstuk 5 laten we zien dat vooral milt en lever VWF kunnen opnemen uit het bloed (figuur 1, hoofdstuk 5). Deze organen bevatten onder andere macrofagen. Macrofagen zijn cellen die in staat zijn grote deeltjes, als resten van cellen en bacteriën op te nemen. Doordat macrofagen grote deeltjes op kunnen nemen zouden ze geschikt zijn voor het verwijderen van het grote plasma-eiwit VWF. Voor deze studie hebben we muizen gebruikt die geen VWF kunnen produceren. Met behulp van deze dieren kunnen we de functie van VWF goed bestuderen. We hebben VWF en FVIII ingespoten in deze muizen en daarna gekeken of in de milt en lever het ingespoten eiwit terug gevonden kan worden. Dit was inderdaad het geval, we konden VWF, FVIII en ook het VWF/FVIII complex terug vinden. We vonden de eiwitten gebonden aan cellen die we als macrofaag konden indentificeren. Hierna hebben we de muizen behandeld met een stofje waardoor hun macrofagen geïnactiveerd werden. In vergelijking met de onbehandelde muizen bevatte het bloed van behandelde muizen hogere VWF concentraties. Dit zou kunnen betekenen dat VWF wordt verwijderd uit het bloed door macrofagen. Om te kijken of deze cellen VWF ook kunnen opnemen en afbreken hebben we proeven gedaan waarbij we eerst macrofagen hebben laten groeien. Vervolgens hebben we VWF toegevoegd en zagen dat de cellen het toegevoegde VWF opnamen en ook afbraken (figuur 6, hoofdstuk 5). Dus macrofagen zijn niet alleen in staat VWF, FVIII en het FVIII/VWF complex te binden, maar in het geval van VWF nemen ze het eiwit ook op om het vervolgens af te breken.

Von Willebrand factor bindt aan Staphylococcus aureus

Een infectie met de bacterie *Staphylococcus aureus* kan ernstige gevolgens hebben. Deze bacterie heeft een aantal eiwitten op zijn oppervlak die ervoor zorgen dat de bacteriën aan plasma-eiwitten en eiwitten in de vaatwand kunnen binden. Hierdoor wordt de bacterie minder snel herkend door het immuunsysteem. Eén van de plasma-eiwitten waaraan *Staphylococcus aureus* kan binden is VWF. Omdat VWF ook aan bloedplaatjes kan binden, komt *Staphylococcus aureus* op deze manier ook in aanraking met bloedplaatjes. Op deze wijze kan *Staphylococcus aureus* endocarditis (een ontsteking van de binnenbekleding van het hart) veroorzaken. Een eiwit dat aanwezig is op het oppervlakte van *Staphylococcus aureus* is het zogenaamde Protein A. De interactie tussen *Staphylococcus aureus* en VWF vindt plaats via dit eiwit. In de studie beschreven in hoofdstuk 7 van dit proefschrift hebben we onderzocht aan welk deel van VWF het Protein A van *Staphylococcus aureus* kan binden. We hebben gevonden dat *Staphylococcus aureus* bindt met het gedeelte tussen helix I en II (figuur 7, hoofdstuk 7) aan de domeinen VWF-D'D3 en VWF-A1 van VWF (figuur 3, hoofdstuk 7).

Samengevat is er aan de ene kant gekeken naar aspekten in het plasma die de concentratie VWF in het bloed beïnvloeden, zoals de O-gekoppelde suikers en cysteine mutaties. Aan de andere kant is het mechanisme dat VWF uit het bloed verwijderd bestudeerd. Met deze nieuwe informatie begrijpen we beter hoe de VWF concentratie in het bloed gereguleerd wordt en kunnen we hopelijk in de toekomst VWF maken dat minder snel door het lichaam wordt geklaard. Dit zou de behandeling van patiënten met VWD en hemofilie A kunnen verbeteren.

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Carina

Curriculum Vitae

Carina van Schooten werd geboren op 11 januari 1978 te Zwolle. In 1997 behaalde zij haar VWO diploma aan het Thomas a Kempis College te Zwolle. Aansluitend studeerde zij Bioprocestechnologie aan de Wageningen Universiteit. Ze voltooide haar wetenschappelijke stage bij de afdeling Celbiologie en Immunologie van de Wageningen Universiteit, te Wageningen. Daarnaast liep ze stage bij het Cancer Genetics Laboratory van de University of Otago in Dunedin, Nieuw-Zeeland en bij N.V. Organon te Oss.

Na het behalen van het doctoraal examen in 2003, begon ze als assistent in opleiding bij de afdeling Klinische Chemie en Haematologie van het Universitair Medisch Centrum Utrecht aan het onderzoek dat heeft geleid tot dit proefschrift, onder begeleiding van Dr. P.J. Lenting, Dr. H.M. van den Berg en Prof. Dr. Ph.G. de Groot.

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