Factor VIII and von Willebrand factor co-delivery by endothelial cells

Eveline Bouwens

Cover: trafficking of green fluorescent Factor VIII leads to formation of FVIII-covered VWF strings

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Factor VIII and von Willebrand factor co-delivery by endothelial cells

Gezamenlijke afgifte van Factor VIII en von Willebrand factor door endotheelcellen

(met een samenvatting in het Nederlands)

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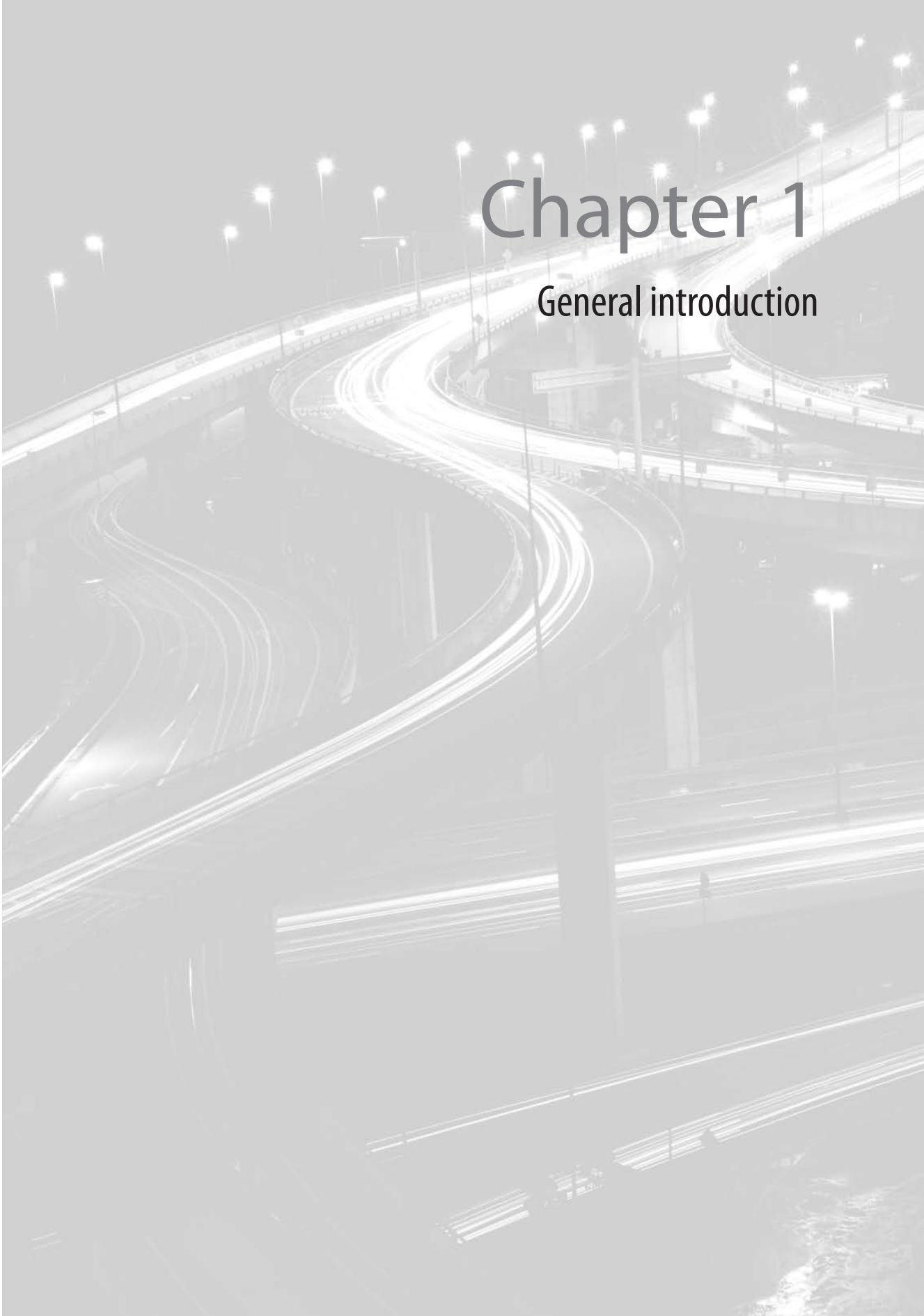
Contents



Chapter 1

General introduction

Outline of this thesis

This thesis addresses the potential of endothelial cells as a vehicle for co-delivery of two major components in hemostasis: factor VIII (FVIII) and von Willebrand factor (VWF). A functional deficiency in either FVIII or VWF results in a bleeding disorder, hemophilia A and von Willebrand's disease (VWD) respectively. Both proteins have their own function in hemostasis but their life cycles are greatly intertwined: since the early seventies it has been known that FVIII and VWF circulate in plasma as a tightly bound protein complex.[1] Assembly with VWF stabilizes the structure of FVIII but also protects FVIII from inactivation and clearance, which increases the half-life of FVIII considerable.[2-4] Surprisingly, the question where the FVIII-VWF complex is formed has remained controversial over decades. As until recently no cell had been identified that secreted both FVIII and VWF, it has been generally assumed that these proteins are synthesized at distinct sites in the body and assemble after release into circulation. However, recent studies show that a subset of endothelial cells has the capacity to endogenously express both FVIII and VWF, suggesting that this cell type may synthesize the FVIII-VWF complex.[5-7] Whether or not FVIII is secreted by the same cell that synthesizes VWF could have major implications for hemophilia A gene therapy. Failure to achieve sustained FVIII levels in gene therapy trials may be the result of targeting the wrong cell for FVIII expression. Perhaps the likelihood of successful gene therapy will increase when FVIII is secreted by endothelial cells. Although complex assembly is extensively studied to optimize FVIII protein replacement, only few groups investigating gene therapy have focused on co-expressing FVIII in cells that synthesize VWF, *i.e.* endothelial cells and megakaryocytes.[8-11] Recently we have shown that FVIII expression in endothelial cells results in co-storage of FVIII with VWF.[12,13] In this study our aim was to explore the potential of endothelial cells as a cellular release-on-demand device for co-delivery of FVIII and VWF. In this first chapter we will review the current knowledge on FVIII and VWF biosynthesis, storage and molecular interactions.

Hemophilia A and von Willebrand's disease

Hemophilia A is an X-linked bleeding disorder caused by the absence, severe deficiency, or dysfunction of coagulation protein FVIII affecting approximately 1 in 5,000 males.[14] Hemophilia A is divided into three categories: mild (6-30% FVIII activity), moderate (2-5% FVIII activity) and severe (<1% FVIII activity).[14] Patients with severe hemophilia have 20 to 30 episodes per year of spontaneous bleedings or excessive bleedings after minor trauma, mostly into joints and muscles.[14] Current treatment of hemophilia A consists of prophylactic or on-demand intravenous administration of either plasma-derived or recombinant FVIII protein.[15] However, treatment is hampered by high cost of FVIII products, limited availability, short half-life of FVIII and inhibitory antibody formation in about 25% of patients.[16-18] It is estimated that only 25% of patients worldwide receive adequate treatment.[19]

Von Willebrand's disease is the most common bleeding disorder with a prevalence of approximately 1%.[20-22] There are several types of VWD each with its own characteristics: type 1 and 3 can be distinguished by a quantitative defect in VWF, whereas qualitative defects characterize type 2 VWD.[23] VWD patients are treated with the vasopressin analogue

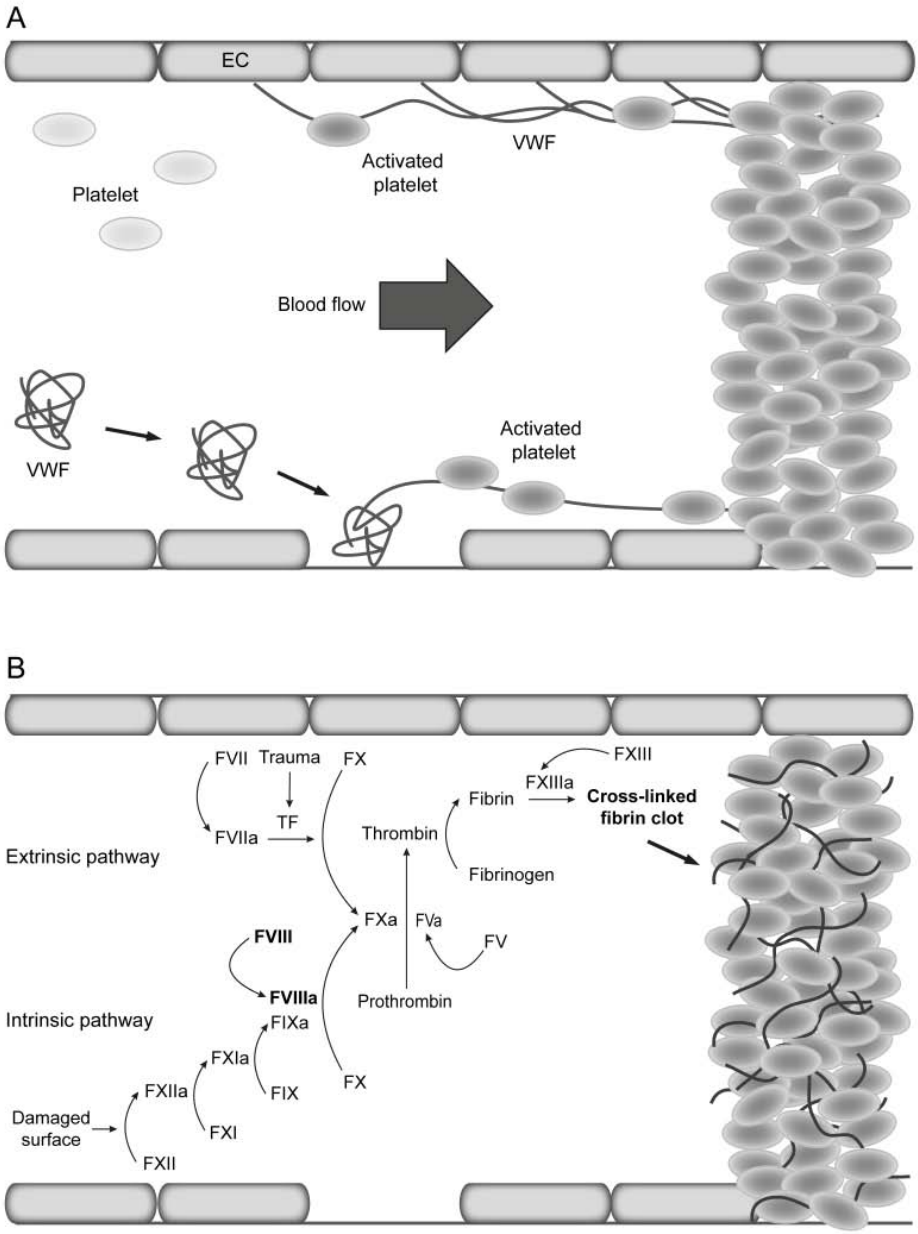


Figure 1. Role of VWF and FVIII in hemostasis. Primary (A) and secondary (B) hemostasis occur in parallel to arrest bleeding. (A) Circulating VWF adheres to the subendothelial matrix that is exposed when the vascular wall is damaged. Blood flow causes bound VWF to unfold and exposes platelet binding sites. In addition, VWF released from stimulated endothelial cells (EC) assembles into strings to which platelet may also adhere. Platelets bound to VWF get activated and start to aggregate, leading to thrombus formation. (B) Schematic representation of the coagulation cascade. Both the intrinsic and extrinsic pathway lead to fibrin clot formation, although the latter is most important. The extrinsic pathway is initiated by exposure of tissue factor (TF) upon vascular damage. FVIII functions as a co-factor for FIXa to activate FX.

desmopressin (DDAVP) that releases storage pools of VWF from endothelial cells. An alternative for patients that do not respond to DDAVP treatment is protein replacement therapy with either VWF alone or in combination with FVIII.[24] A subclass of type 2 VWD, known as type 2N or the Normandy variant, has a strongly reduced binding affinity for FVIII resulting in FVIII deficiency.[23] Failure in FVIII-VWF complex formation leads to rapid clearance of FVIII from circulation, which gives a phenotype that closely resembles hemophilia A. VWF levels are normal but bleeding episodes occur due to low circulating FVIII levels (<10% FVIII activity) despite that FVIII itself is produced at normal rate and is fully functional. This emphasizes the importance of FVIII-VWF complex assembly for restoration of circulating FVIII levels.

Role of factor VIII and VWF in bleeding arrest

Vascular injury simultaneously leads to formation of a platelet plug and blood coagulation. VWF plays a key role in primary hemostasis or platelet plug formation whereas FVIII functions as a co-factor in thrombin generation. When a vessel wall is damaged, circulating VWF adheres to the uncovered subendothelial matrix and endothelial cells release additional VWF. Blood flow imposes shear stress on VWF and causes it to unfold, allowing platelets to attach to newly exposed binding sites on the VWF molecule.[25,26] Platelets bound to VWF get activated and start to aggregate, which eventually leads to formation of a thrombus that occludes the vessel (Figure 1A).

Besides its function in platelet plug formation, VWF serves as a natural carrier protein for FVIII by increasing the circulatory half-life of FVIII from 2 hours to 12-14 hours.[27] Since FVIII is bound to VWF in plasma, VWF adhesion to exposed subendothelial matrix will also accumulate FVIII at sites of vascular injury giving a local rise in FVIII levels.[25] When FVIII is activated by thrombin or factor Xa (FXa), the high-affinity interaction between FVIII and VWF is lost and the FVIII-VWF complex dissociates.[28] In presence of Ca^{2+} ions and a negatively charged membrane activated FVIII (FVIIIa) assembles with factor IXa (FIXa) to form a factor X-activating (FXase) complex that cleaves FX to its active form. Binding of FVIIIa increases FIXa protease activity by several orders of magnitude.[28] Activation of FX ultimately results in formation of fibrin that stabilizes the thrombus (Figure 1B).

Factor VIII and VWF structure

FVIII is synthesized as a preprotein of 2351 amino acids comprising a signal peptide of 19 amino acids and a mature protein of 2332 residues organized as A1-a1-A2-a2-B-a3-A3-C1-C2 (Figure 2A).[27,29] The A domains are bordered by short regions, a1 (residues 337-372), a2 (residues 711-740) and a3 (residues 1649-1689), that contain clusters of acidic residues. The A domains are responsible for binding to thrombin, FIXa and FX in the FX-activating complex.[30-32] Both C domains have been implicated in phospholipid binding as well as binding to VWF, thrombin and FXa.[30,33-38] The function of the B domain is not fully understood but the domain is dispensable for FVIII activity.[39]

FVIII is cleaved intracellular in the B domain at residues Arg1313 and Arg1648. This proteolysis yields a heterodimeric FVIII protein with a 90-220 kDa heavy chain (A1-a1-A2-a2)

non-covalently attached to a 80 kDa light chain (a3-A3-C1-C2) by a metal-dependent interaction in the A1 and A3 domain (Figure 2B).[40] Activation by thrombin or FXa through cleavage at the a1 (Arg372-Ser373), a2 (Arg740-Ser741) and a3 (Arg1689-Ser1690) regions, creates a heterotrimer consisting of A1, A2, A3-C1-C2. The A2 domain is only weakly associated to the other domains, which makes the heterotrimer very unstable. Spontaneous dissociation of the A2 domain therefore leads to rapid inactivation of FVIII.[41]

VWF is synthesized as a prepropolypeptide of 2813 residues consisting of a propeptide (D1-D2) and a mature VWF monomer (D'-D3-A1-A2-A3-D4-B1-B2-B3-C1-C2-CK) of approximately 250 kDa (Figure 2C).[42] Binding sites for FVIII, P-selectin and propeptide are all located in the D'-D3 region of VWF.[43] The A1 and A3 domains are responsible for binding to collagen in the subendothelial matrix, whereas the A1 domain can also bind to GP1bα on platelets and to heparin.[43,44] A cleavage site for the metalloproteinase ADAMTS13 is located at Tyr1605-Met1606 in the A2 domain.[45] The C1 and C2 domains can bind integrin $\alpha_{IIb}b_3$ which is present on several cell types, including platelets and endothelial cells.[45,46]

Factor VIII biosynthesis and secretion

FVIII is mainly synthesized in the liver but FVIII mRNA has also been detected in spleen, lymph nodes, pancreas, kidney, brain, heart, lung, testis and muscle.[47,48] The major FVIII producing cell in the liver has not been identified, although hepatocytes and liver sinusoidal endothelial cells (LSECs) both have been shown to produce FVIII.[5,49,50] Several lines of evidence indicate that FVIII is also secreted by an extrahepatic source. Liver transplantation from a normal donor to a hemophilic recipient restores FVIII levels in circulation but DDAVP infusion does not release stored FVIII.[51] This indicates that the DDAVP-inducible FVIII storage pool is produced or stored outside the liver. Recent publications have shown that a subset of endothelial cells synthesize FVIII; microvascular endothelial cells from lung, heart, intestine and skin, endothelial cells from pulmonary artery, and hepatic sinusoidal endothelial cells have all been shown to secrete FVIII.[6,7,52] On the other hand, endothelial cells exist that do not express FVIII, such as human umbilical vein endothelial cells (HUVEC). These do not contain FVIII mRNA, indicating that absence of FVIII production in these endothelial cells results from lack of mRNA rather than a defect in the FVIII secretion pathway.[6]

FVIII is poorly secreted by cells, which seems to result not only from low mRNA expression but also from inefficient secretion.[53] FVIII undergoes substantial intracellular processing, including *N*- and *O*-linked glycosylation and sulfation of certain tyrosines. Within the ER FVIII interacts with a number of chaperone proteins, which is responsible for retention of FVIII in the ER and subsequent limited transport to the Golgi apparatus.[53,54] Expressing FVIII in cells that have the machinery to endogenously synthesize this protein, such as endothelial cells, may therefore give a higher protein yield than expression of FVIII in other cell types.

VWF biosynthesis, storage and secretion

VWF is a multimeric glycoprotein solely found in platelets and endothelial cells.[55-57] VWF is synthesized as a monomer but undergoes dimerization in the ER through disulfide bridge

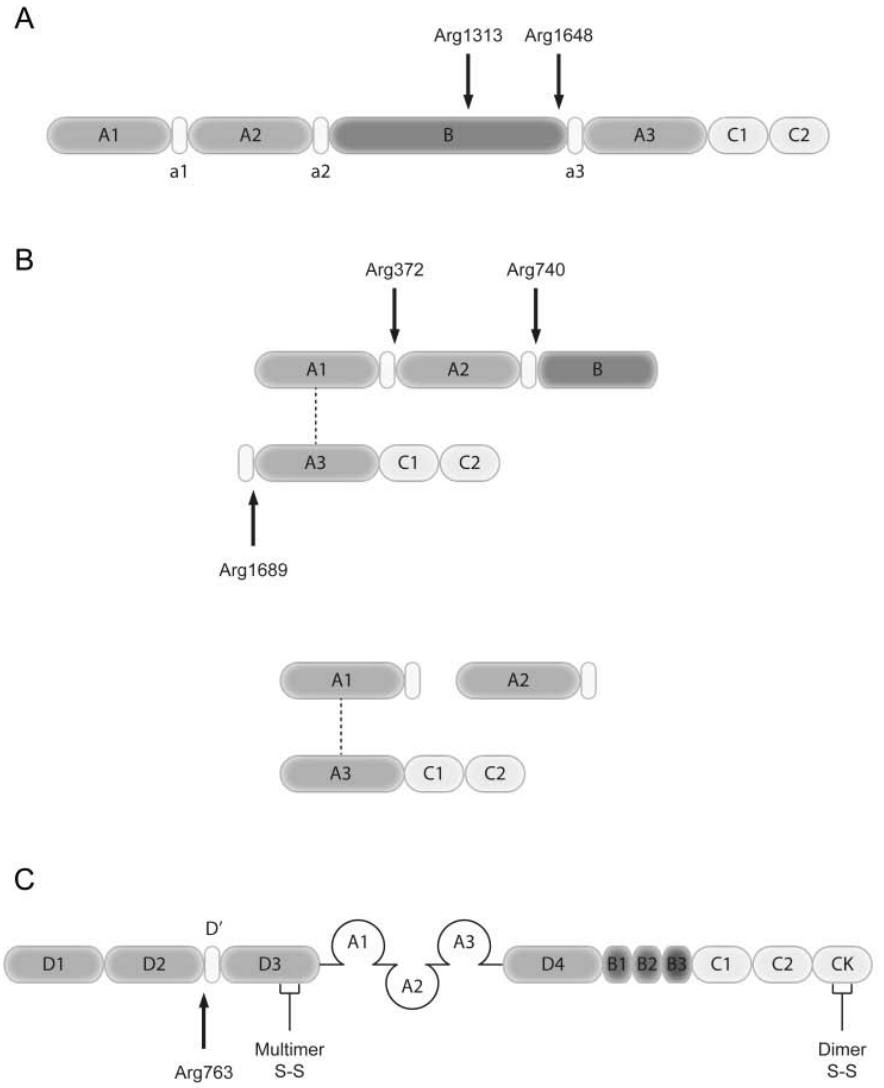


Figure 2. Schematic representation of FVIII and VWF. (A) Domain structure of FVIII with B domain cleavage sites indicated by arrows. (B) Circulating FVIII consist of a heavy (A1-A2-B) and light chain (A3-C1-C2) that are non-covalently associated between the A1-A3 domains through a metal ion-dependent interaction. Activation of FVIII at the indicated thrombin cleavage sites results in formation of a heterotrimer, in which the A2 domain is weakly associated with the A1 domain (C) Domain structure of VWF with the furin cleavage site behind the propeptide indicated with an arrow. Dimerization occurs via disulfide bond formation in the CK domains, multimerization is mediated through disulfide linkage in the D'-D3 regions.

formation in the CK domains. After dimerization furin cleaves behind the propeptide to release it from the VWF dimer.[58] Additional multimerization between D3 domains takes place in the *trans*-Golgi network (TGN), which is facilitated by the propeptide.[59-62] Transport of exceptionally large proteins from the ER to TGN is difficult and therefore VWF multimerization probably takes place after transport to the TGN, while dimers are formed in the ER.[62]

VWF expressed in endothelial cells is stored into cigar-shaped, secretory vesicles known as Weibel-Palade bodies (WPBs). During WPB formation and maturation VWF multimers assemble into long, helical tubules, condensing VWF length in WPBs approximately 50-fold.[63] Smaller VWF multimers are constitutively and basally secreted into circulation, while higher multimers are only released upon agonist-induced exocytosis of WPBs.[64,65] These high molecular weight (HMW) multimers are more active in binding platelets and the subendothelial matrix than smaller multimers.[65,66]

Factor VIII interaction with VWF

FVIII binds with high affinity to VWF with a K_d in the subnanomolar range (0.2-0.5 nM)[67-69] The VWF binding region on FVIII comprises an extended surface, spanning multiple domains located in the light chain.[70-72] The main interaction between FVIII and VWF takes place in the acidic a3 region, in particular through the sulfated Y1680.[67,73,74] However, peptides representing the 1673-1689 region are not able to compete for FVIII-VWF complex formation indicating that also other regions in FVIII contribute to the high-affinity binding between FVIII and VWF.[74] Indeed, residues in the C1 and C2 domain provide secondary binding sites for VWF. Multiple hemophilic mutations in the C1 domain result in reduced binding of FVIII to VWF with the mutation S2119Y giving the largest reduction in VWF binding.[75] The separately expressed C2 domain has been shown to bind VWF[76] and a hydrophobic loop in the C2 domain comprising M2199, F2200, L2251 and L2252 is of importance for binding to VWF and phospholipids upon dissociation of the FVIII-VWF complex.[77,78]

Factor VIII co-storage with VWF

FVIII endogenously expressed by endothelial cells is partly co-stored with VWF in WPBs.[6] Co-storage of FVIII in (pseudo-)WPBs is even more distinct when FVIII is over-expressed in HUVEC or is co-expressed together with VWF in cells with a regulated secretory pathway, like human embryonic kidney cells (HEK293).[12,13,79] An apparent discrepancy between association of FVIII and VWF in plasma and co-trafficking to secretory vesicles has been revealed by expressing FVIII and VWF mutants defective in complex assembly in cells that synthesize and store VWF. These experiments demonstrate that FVIII Y1680F, with strongly reduced VWF binding capacity in solid-phase and pseudo-equilibrium binding assays, is still able to target to WPBs.[13] Also co-expression of FVIII with several VWD 2N VWF variants does not influence FVIII targeting to WPBs.[12] This raises the possibility that FVIII targets to WPBs by a low-affinity interaction with VWF through a mechanism that is presently unclear.

Cellular delivery of FVIII-VWF complex

Limitations in FVIII replacement therapy provide a rational to explore gene therapy as a cure for hemophilia A. Most gene delivery studies have targeted FVIII gene expression to hepatocytes since the liver is the main site of FVIII synthesis. Although positive results were obtained in small animals[80-82] and hemophilic dogs[83,84], sustained FVIII levels in patients have not been accomplished so far.[85-88] Bottlenecks that arise from clinical trials include inhibitor formation, low or non-detectable FVIII levels and toxicity of the vector. A way to circumvent several of these issues could be to target FVIII to cells that express VWF. Co-storage of FVIII with VWF has the potential of directly releasing FVIII-VWF complex. As FVIII in complex with VWF has prolonged circulation time, is protected from proteolytic inactivation and immune response, this may have an advantage over simple release of FVIII from hepatocytes or other cell types that do not synthesize VWF.

Scope of this thesis

On a cellular level many issues concerning FVIII and VWF co-delivery by endothelial cells remain poorly understood. For instance it is unclear how much of the FVIII produced by endothelial cells is co-stored with VWF in WPBs, a question of high importance when the objective is co-delivery of these proteins. In **chapter 2** we demonstrate that endothelial cells can store a considerable amount of FVIII in WPBs, which provides a FVIII-VWF storage pool that can be released upon agonist-induced WPB exocytosis. In addition, a striking change in WPB morphology was observed when FVIII was expressed in endothelial cells. The signal for sorting of FVIII to WPBs has not been resolved and the role of VWF herein remains controversial. **Chapter 3** addresses the sorting efficiency of several hemophilic FVIII variants that display reduced VWF binding. Besides that the ability to sort to WPBs is not affected by the hemophilic mutations, also the relative amount of FVIII stored in WPBs is independent of the various mutations. We provide evidence that sorting of these FVIII variants may be dependent on their ability to interact with VWF at low pH. Moreover, WPBs containing FVIII variants are indistinguishable from WPBs storing normal FVIII in that they become round. As the tubular organization of VWF in WPBs is considered essential for its ability to bind platelets, we studied the cause of this morphologic change and its effect on VWF storage and functionality in **chapter 4**. We show that FVIII not only disrupts VWF tubules but also decreases platelet adhesion to VWF strings from FVIII-expressing endothelial cells. **Chapter 5** describes the interaction of FVIII with VWF after release from rounded WPBs. We demonstrate that WPB-derived FVIII is capable of binding to VWF strings secreted by these cells. Moreover, although the typical high-affinity binding to VWF via tyrosine 1680 is not necessary for VWF binding of endogenously released FVIII, this interaction is important for association of exogenously added FVIII with VWF strings. As these results indicate that intracellular FVIII-VWF assembly differs from extracellular FVIII-VWF binding, we studied the domains involved in sorting of FVIII to WPBs in **chapter 6**. We show that both C domains of FVIII are involved in FVIII trafficking to WPBs presumably by an interaction with VWF. Finally, in **chapter 7** we discuss the potential of endothelial cells to deliver FVIII together with VWF as a treatment for hemophilia A.

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Chapter 2

Storage and regulated secretion of factor VIII in blood outgrowth endothelial cells

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Abstract

Gene therapy provides an attractive alternative for protein replacement therapy in hemophilia A patients. Recent studies have shown the potential benefit of directing factor (F)VIII gene delivery to cells that also express its natural carrier protein von Willebrand factor (VWF). In this study, we explored the feasibility of blood outgrowth endothelial cells as a cellular FVIII delivery device with particular reference to long-term production levels, intracellular storage in Weibel-Palade bodies and agonist-induced regulated secretion. Human blood outgrowth endothelial cells were isolated from peripheral blood collected from healthy donors, transduced at passage 5 using a lentiviral vector encoding human B-domain deleted FVIII-GFP and characterized by flow cytometry and confocal microscopy. Blood outgrowth endothelial cells displayed typical endothelial morphology and expressed the endothelial-specific marker VWF. Following transduction with a lentivirus encoding FVIII-GFP, 80% of transduced blood outgrowth endothelial cells expressed FVIIIGFP. Levels of FVIII-GFP positive cells declined slowly upon prolonged culturing. Transduced blood outgrowth endothelial cells expressed 1.6±1.0 pmol/1×10^6 cells/24h FVIII. Morphological analysis demonstrated that FVIII-GFP was stored in Weibel-Palade bodies together with VWF and P-selectin. FVIII levels were only slightly increased following agonist-induced stimulation, whereas a 6- to 8-fold increase of VWF levels was observed. Subcellular fractionation revealed that 15-22% of FVIII antigen was present within the dense fraction containing Weibel-Palade bodies. We conclude that blood outgrowth endothelial cells, by virtue of their ability to store a significant portion of synthesized FVIII-GFP in Weibel-Palade bodies, provide an attractive cellular on-demand delivery device for gene therapy of hemophilia A.

Introduction

Hemophilia A is an X chromosome-linked bleeding disorder affecting 1-2 in 10,000 males. It results from quantitative or qualitative defects of blood coagulation factor (F)VIII. Current treatment includes protein replacement therapy with plasma-derived or recombinant FVIII concentrates.[1] However, due to its short half-life in circulation ($t_{1/2}$ approximately 12 h), treatment requires frequent intravenous infusions of FVIII concentrates. Gene therapy or cell-based approaches have the potential to provide a life-long, cost-effective cure for hemophilia A.[2] A number of clinical trials for hemophilia A gene therapy have been performed.[3,4] So far, limited clinical efficacy of gene delivery approaches has been observed. This is primarily caused by limitations in obtaining sufficiently high levels of FVIII in the circulation. This may be partly due to the fact that the exact cellular site of FVIII biosynthesis has not been clearly identified. Recent studies have suggested that it may be beneficial to express FVIII in cells that also express its natural carrier protein von Willebrand factor (VWF).[5-11] In the circulation, VWF protects FVIII from premature clearance and proteolytic degradation by virtue of its ability to bind to it with high affinity.[12-14] VWF is expressed in megakaryocytes and vascular endothelial cells.[15] In endothelial cells, synthesis of VWF drives the formation of elongated cigar-shaped storage organelles called Weibel-Palade bodies (WPBs) that release their content upon agonist-induced stimulation.[15-18] Expression of FVIII in endothelial cells results in storage of FVIII in WPBs.[6,19,20] Co-storage of the VWF-FVIII complex in secretory granules and subsequent release of the VWF-FVIII complex upon agonist-induced stimulation has the potential of secreting large amounts of FVIII at sites of vascular injury as well as directly increasing FVIII half-life by protecting FVIII from premature clearance and proteolytic degradation. Endothelial (progenitor) cells have been used for cell-based therapy as well as for gene therapy. Studies in hemophilia A mice have demonstrated that transplantation of liver sinusoidal endothelial cells can correct the hemophilic phenotype.[9,21] In addition, transplantation of genetically modified BOECs intravenously[10,22] or implanted subcutaneously in a Matrigel™ scaffold[10] results in long-term therapeutic levels of FVIII. The above mentioned studies have demonstrated proof of principle that endothelial cells are capable of long-term synthesis and secretion of large amounts of FVIII. However, quantitative aspects of FVIII secretion and storage in endothelial cells have not yet been explored. Here, we analyzed the intracellular routing of FVIII and VWF in genetically modified BOECs using fluorescently tagged FVIII. We demonstrate that part of the synthesized FVIII-GFP is present within WPBs, providing a reservoir of FVIII that can be released following vascular perturbation.

Materials and methods

Materials

All chemicals used were of analytical grade. Endotoxin Free Plasmid Isolation kits were from Qiagen (Hilden, Germany). Ficoll-Paque Plus was from GE Healthcare (Uppsala, Sweden). Fetal Calf Serum (FCS) was from Hyclone (Logan, UT, USA). One shot *Stbl3* chemically competent cells, Hank's Balanced Salt Solution (HBSS), antibiotic/antimycotic, trypsin, DMEM (4.5 g/L glucose), RPMI-1640 and M199-Hepes were obtained from Invitrogen (Breda, the Netherlands). DNA modifying enzymes were from Fermentas (St Leon-Rot, Germany). Streptomycin and penicillin were from BioWhittacker (Verviers, Belgium). Endothelial Cell Medium-2 (EGM-2) was obtained from Lonza (Walkersville, MD, USA). Collagen type 1 rat tail was from BD Biosciences (Upssala, Sweden). Phorbol 12-myristate 13-acetate (PMA), calcium ionophore A23187, epinephrine, thrombin, isobutylmethylxanthine (IBMX) and brefeldin A (BFA) were from Sigma- Aldrich (St-Louis, MO, USA). Culture plates, cell factory (6320 cm^2) and microtiterplates (Maxisorp) were from Nunc (Roskilde, Denmark).

Production of viral vectors

The lentiviral (LV) packaging system consists of three constructs encoding gag/pol (pMDL.RRE), vesicular stomatitis virus glycoprotein envelope (pCMV-VSV-G) and rev (pRSV-Rev).[23] The self-inactivating lentiviral vector construct pLV CMV-GFP has been described before.[24] A lentiviral self-inactivating vector encoding human B-domain deleted FVIII under control of the CAG promoter consisting of the chicken β-actin promoter, CMV enhancers and a large synthetic intron has been previously described.[25] FVIII-GFP with GFP replacing the B-domain in pcDNA3.1(+) has also been described before.[20] pLV CAG-FVIII-GFP was created by ligation of fragment *NheI-NotI* from FVIII-GFP and fragment *XbaI-NotI* from the lentiviral vector in presence of a linker sequence *XbaI*-TCTGCTAACCATGTTCATGCCTTCTTCTTTTTCCTACAGGCTAGC-*NheI*. Lentiviral vector was produced as previously described[26] with minor modifications. Briefly, 293T cells were seeded in a 6320 cm^2 cell-factory and transfected with 1.9 mg/l lentiviral vector, 0.82 mg/l pMDL.RRE, 0.32 mg/l pRSV-Rev and 0.44 mg/l pCMV-VSV-G using the calcium phosphate co-precipitation method. Twenty-four hours after transfection, medium was exchanged for fresh medium containing 100 mg/l sodium butyrate. Viral supernatant was collected after an additional 48 hour incubation period, filtered through a 0.45 µm Durapore (PVDF) membrane (Millipore, Billerica, MA, USA), and concentrated by centrifugation at 50,000*g* for two hours. Lentiviral vectors were resuspended in HBSS and stored in aliquots at -80°C. Vector titers were determined by transduction of 1×10^5 293T cells with serially diluted vector followed by flow cytometry. Titers were calculated with the following formula:[25] transducing units (TU) per milliliter = $n \times (P/100)/V$, where n = number of cells at time of transduction (10^5), P = percentage of GFP-positive cells, and V = volume of viral preparation added in milliliters.

Blood outgrowth endothelial cell isolation and transduction

BOECs were isolated essentially as described[22] from 50 ml venous blood donated by healthy volunteers. Passage five cultured BOECs (2.5×10^4) were transduced following a single exposure to pLV-CMV-GFP (MOI 10) or pLV-CAG-FVIII-GFP (MOI 7.5) in the presence of 8 µg/ml polybrene (Janssen Chimica, Beerse, Belgium) and centrifuged for 90 min at 300*g* and 32°C. After a four hour total incubation time, medium was refreshed, and transduced cells were further cultured using standard BOEC cell culture procedures. To determine steady state production levels, conditioned medium was centrifuged for 10 min at 10,000*g*, and supplemented with 10 mM benzamidine.

Flow cytometry

Percentage of GFP positive cells was determined by flow cytometry. Cells were washed twice with HBSS, lifted with 0.05% trypsin-EDTA and were resuspended in 1% (w/v) bovine serum albumin (BSA) (Albumin Fraktion V, Merck, Darmstadt, Germany) in phosphate buffered saline (PBS) supplemented with 10% (v/v) Cellfix (BD Biosciences, Uppsala, Sweden). Flow cytometry was performed using the LSR II (BD Biosciences, Uppsala, Sweden). The acquired data were analyzed with FACSDiva software (BD Biosciences, Uppsala, Sweden).

Quantification of FVIII and von Willebrand factor

FVIII antigen was quantified using an anti-light chain ELISA. Monoclonal anti-FVIII light chain antibody CLB-CAg12[27] was coated into a 96-well microtiterplate (5 µg/ml; 100 µl/well) in 50 mM $NaHCO_3$ (pH 9.8) for at least 16 h at 4° C. Plates were washed with PBS, 0.1% (v/v) Tween-20 (pH 7.4). Samples were diluted in 2% (v/v) human serum albumin (HSA) (Cealb, Sanquin, Amsterdam, the Netherlands), 1 M NaCl, 50 mM Tris (pH 7.4) and incubated with the immobilized antibody for two hours at 37°C. Peroxidase-labeled monoclonal anti-FVIII light chain antibody CLB-CAg117[28] was used to detect bound FVIII light chain. Normal human pooled plasma was used as standard. FVIII activity was quantified using a chromogenic assay according to the manufacturer's instructions (Chromogenix, Milan, Italy).

Human VWF antigen was quantified using an ELISA. Monoclonal anti-VWF antibody CLB-RAg20[29] was coated into a 96-well microtiterplate (1 µg/ml; 100 µl/well) in 50 mM $NaHCO_3$ (pH 9.8) for at least 16 h at 4° C. Plates were washed with 0.1% (v/v) Tween-20, phosphate buffered saline (PBS) (pH 7.4). Samples were diluted in 0.1% (v/v) Tween-20, 1% (w/v) bovine serum albumin (BSA) (Albumin Fraktion V, Merck, Darmstadt, Germany), PBS (pH 7.4) and incubated with the immobilized antibody for two hours at 37°C. Peroxidase-labeled polyclonal rabbit anti-human VWF antibody (DAKO, Glostrup, Denmark) was used to detect bound VWF. Normal human pooled plasma was used as standard.

Immunofluorescence microscopy

Non-transduced BOECs were grown on 1 cm-diameter gelatin-coated glass coverslips. Confluent cells were fixed with PBS/3.7% paraformaldehyde (PFA) and prepared for immunofluorescence analysis as described.[30] Monoclonal antibody CLB-RAg20[29] was used to visualize VWF. Rabbit polyclonal antibody anti-human CD62-P (BD PharMingen, San Diego, CA, USA) was used to visualize P-selectin. Monoclonal antibody CLB-HEC75[31] was used to visualize PECAM-1 (CD31). Alexa 594- and Alexa 633-conjugated secondary antibodies were from Invitrogen (Breda, the Netherlands). Cells were embedded in Vectashield mounting medium (Vector Laboratories, Burlington, CA, USA) and viewed by Confocal Laser Scanning Microscopy using a Zeiss LSM510 (Carl Zeiss, Heidelberg, Germany).

Stimulation of Weibel-Palade body exocytosis

BOECs were plated at 1-2×10^5 cells/well in collagen-coated 10 cm^2 wells. Conditioned medium was refreshed every other day until confluency. The plates were washed twice with serum-free (SF) medium (50% RPMI-1640 and 50% M199-Hepes) supplemented with 1% (v/v) HSA (Cealb, Sanquin, Amsterdam, the Netherlands), 0.3 mg/ml L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. After incubation with SF medium for one hour, the cells were stimulated for one hour with SF medium containing 50 ng/ml phorbol 12-myristate 13-acetate (PMA), 10 µM calcium ionophore A23187, 10 µM epinephrine plus 100 µM IBMX, or 1 U/ml thrombin. The

supernatant was collected, centrifuged for 10 min at 10,000*g*, supplemented with 10 mM benzamidine and stored at -20°C until use. Cells were subsequently washed and prepared for morphological analysis as described above. FVIII-GFP transduced BOECs were plated at 1×10^5 cells/well in collagen-coated 6-well plates and stimulated with 50 ng/ml PMA as described above.

Inhibition of constitutive release by brefeldin A

Brefeldin A (BFA) treatment of FVIII-GFP transduced BOECs was performed essentially as previously described.[32] Confluent cells were washed twice with SF medium and subsequently incubated for five hours with SF medium supplemented with 5 µM BFA. Medium was collected at several time points and fresh SF medium with BFA was added. Culture supernatants were centrifuged for 10 min at 10,000*g*, supplemented with 10 mM benzamidine and stored at -20°C until further use. FVIII antigen and activity and VWF antigen were determined as described above.

Subcellular fractionation

Subcellular fractionation using Percoll density gradient centrifugation was performed as described with minor modifications.[33] Briefly, BOECs were cultured in two 175 cm^2 culture flasks until they reached confluence. Cells were washed once with warm HBSS, trypsinized and centrifuged for 10 min at 300*g* at 4°C. Cells were suspended in 0.25 M Sucrose, 1 mM EDTA, 20 mM Tris (pH 7.4) and supplemented with 100 µl protease inhibitor cocktail (Sigma-Aldrich, St-Louis, MO, USA). Cells were homogenized by 20 strokes in a ball-bearing homogenizer (Isobiotec, Heidelberg, Germany) with a 14-micron clearance. The homogenate was centrifuged for 10 min at 300*g* at 4°C. The perinuclear supernatant (PNS) was loaded on a gradient consisting of 40% (v/v) Percoll in 0.42 M Sucrose, 1.68 mM EDTA, 33.7 mM Tris (pH 7.4). The Percoll gradient was centrifuged for 30 min at 100,000*g* and 4°C. Fractions of 1 ml were collected from the bottom up and stored at -20°C until further use. FVIII and VWF antigen levels were quantified as described above.

Statistical analysis

Student's *t* test was performed with Graphpad Prism version 4.03 (Graphpad Software, San Diego, USA).

Results

Regulated agonist-induced secretion of WPBs from blood outgrowth endothelial cells

To study the feasibility of BOECs as a cellular delivery device we isolated BOECs from peripheral blood of healthy individuals. Phenotypic characterization revealed that BOECs displayed typical endothelial 'cobblestone' morphology (Figure 1Ai). Cells were uniformly positive for PECAM-1 (CD31) (Figure 1Aii). Staining for VWF revealed the typical rod-shaped structures representing WPBs (Figure 1Aii). To investigate the possibility of using WPBs as an on-demand protein storage pool, we studied the extent of agonist-induced regulated secretion of VWF from BOECs. Stimulation with various known agonists of WPB exocytosis in HUVEC resulted in release of WPBs and secretion of VWF in the conditioned medium. Stimulation with PMA, calcium ionophore A23187, epinephrine and thrombin resulted respectively in a 6-, 4-, 3.5- and 2-fold increase of the VWF concentration in the conditioned medium compared to the non-stimulated control (Figure 1B). Confocal analysis demonstrated that the non-stimulated BOECs contained numerous WPBs (Figure 1C). In comparison, BOECs stimulated with PMA contained hardly any WPBs (Figure 1C), indicating that the increase in VWF antigen in the conditioned medium resulted from VWF release from the majority of WPBs. We observed clustering of the WPBs in the perinuclear region of the cell after stimulation with epinephrine (Figure 1C; arrow), which is consistent with previous studies on epinephrine-stimulated HUVECs.[30]

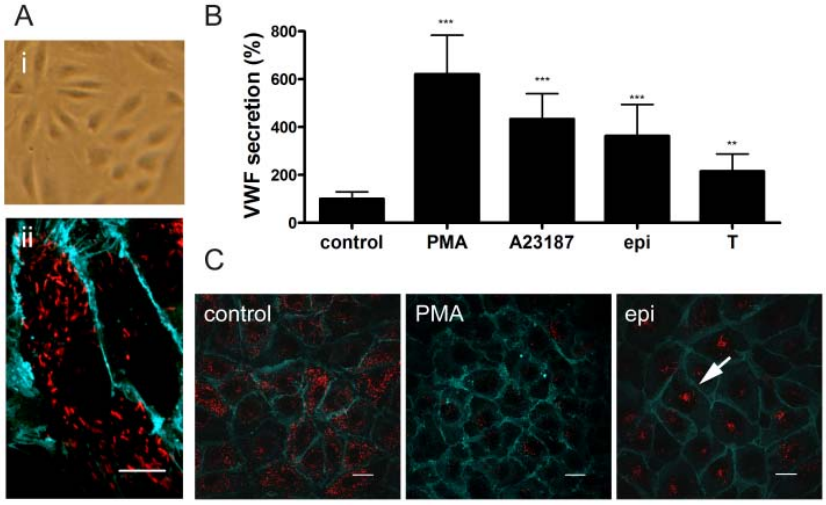

Figure 1. Blood outgrowth endothelial cells as on-demand storage pool. (A) Phenotypic characterization of BOECs revealed a typical endothelial morphology (i). Cells were stained for VWF, shown in red, and for PECAM-1, shown in blue (ii). Scale bar represents 10 µm. (B) BOECs were stimulated for 1 h with 50 ng/mL PMA, 10 µM A23187, 10 µM epinephrine plus 100 µM IBMX (epi), or 1 U/mL thrombin (T). VWF antigen in the conditioned medium was quantified by ELISA. Each value represents the mean±sd of six experiments. **p<0.01, ***p<0.001. (C) Stimulated BOECs were stained for VWF (red). Cells were stained for PECAM-1 using monoclonal antibody CLB-HEC75, shown in blue. Scale bar represents 10 µm. Non-stimulated, control cells contained numerous WPBs (left panel), whereas PMA-stimulated BOECs had released nearly all WPBs (middle panel). After stimulation with epinephrine (right panel) WPBs clustered in the perinuclear region of the cells (arrow).

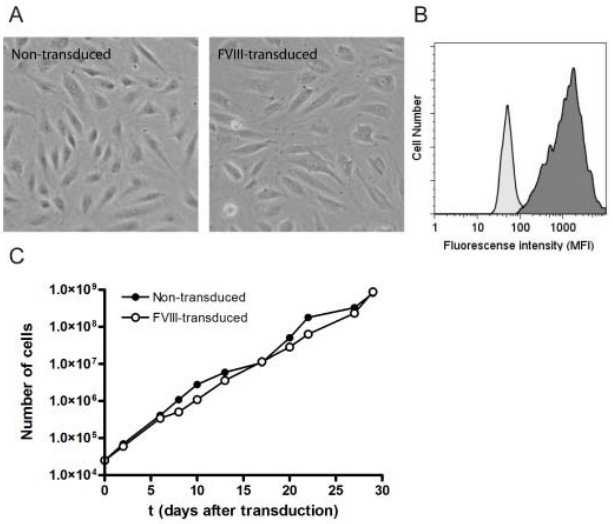

Figure 2. Lentiviral transduction of blood outgrowth endothelial cells with FVIII. BOECs were transduced by spinoculation with lentiviral vectors encoding for FVIII-GFP (MOI 7.5) or GFP (MOI 10). (A) The morphology of non-transduced BOECs and FVIII-transduced BOECs was characterized using light microscopy. (B) Growth rate of non-transduced (closed circles) and FVIII-transduced (open circles) BOECs was determined. (C) Ten days after transduction, the percentage of positive cells was determined by flow cytometry. Approximately 80% of the FVIII-transduced BOECs were positive for FVIII-GFP. The results displayed are representative of at least two different experiments.

Lentiviral FVIII-GFP transduction of Blood outgrowth endothelial cells

BOECs were transduced at passage 5 using a lentiviral vector encoding GFP or human B-domain deleted FVIII-GFP. The transduction did not affect the morphology (Figure 2A) or the growth rate of the transduced BOECs (Figure 2B), as compared to the non-transduced BOECs. Ten days after transduction, FVIII-transduced BOECs were approximately 80% positive, as determined by flow cytometry (Figure 2C). This percentage declined slightly to 60% over 31 days of culture (data not shown). Thus, the majority of the cells showed sustained expression of FVIII-GFP.

Quantification of FVIII and VWF expression by genetically modified BOECs

The production levels of FVIII and VWF of genetically modified BOECs were assessed by quantification of VWF and FVIII antigen levels present in conditioned medium. FVIII-GFP-transduced BOECs expressed on average 1.6±1.0 pmol/1×10^6 cells/24 h FVIII light chain antigen. To assess the functionality of FVIII secreted by BOECs, we determined the activity/antigen ratio. The activity/antigen ratio of the secreted FVIII was 0.9±0.2 after one hour incubation. These data suggest that the FVIII secreted by BOECs is fully active. This is in agreement with published data from Herder *et al*.[34] who observed mean activity/antigen ratios of 0.54 to 0.83 after transduction of cord blood derived endothelial progenitor cells (CBECs) with a lentiviral vector encoding FVIII. The activity/antigen ratio declined to 0.2±0.1 after 24 hour incubation. The FVIII-transduced BOECs secreted VWF at 0.45±0.23 pmol/10^6 cells/24 h. These levels were similar for non-transduced BOECs (0.49±0.35 pmol/10^6 cells/24 h), indicating that lentiviral transduction and production of high levels of FVIII did not affect VWF synthesis (data not shown).

Co-localization of FVIII and von Willebrand factor in Weibel-Palade bodies

The intracellular localization of FVIII in transduced BOECs was assessed by confocal microscopy. In the vast majority of the cells, FVIII co-localized with VWF in WPBs that retained the ability to

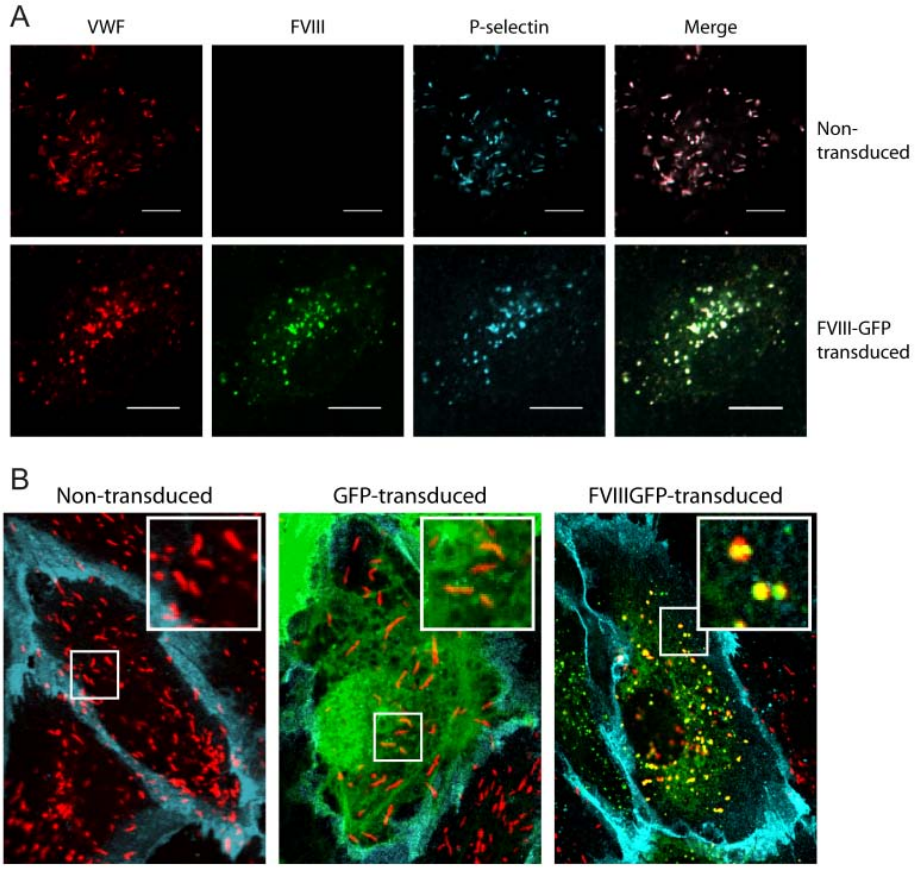

Figure 3. Intracellular localization of FVIII in Weibel-Palade bodies. (A) Non-transduced and FVIII-transduced BOECs were stained for VWF (red) and P-selectin (blue). FVIII co-localizes with VWF and P-selectin in the majority of WPBs. The scale bars represent 10 µm. (B) To visualize morphology of WPBs non-transduced, GFP-, and FVIIIGFP-transduced BOECs were stained for VWF (red) and PECAM-1 (blue). The inset demonstrates elongated, cigar-shaped WPBs in the absence of FVIII and round WPBs in cells expressing FVIII. The scale bars represent 10 µm.

recruit the transmembrane protein P-selectin (Figure 3A). As we have described before in HUVEC[35], storage of FVIII changed the morphology of the WPBs from elongated to round vesicles (Figure 3B insets). As expected, GFP alone did not co-localize with VWF (Figure 3B). Although the shape of the vesicles was altered by the presence of FVIII, WPBs were still able to recruit the transmembrane protein P-selectin, thereby confirming that FVIII-containing vesicles correspond to authentic WPBs (Figure 3A).

Exocytosis of Weibel-Palade bodies from FVIII-transduced BOECs

We subsequently addressed the amount of FVIII released from transduced BOEC in response to agonists that provoke release of WPBs. Stimulation of FVIII-transduced BOECs with PMA resulted in an 8-fold increase of VWF in the culture medium compared to the non-stimulated control (Figure 4A). In contrast to VWF, there was only a slight 1.3-fold increase of FVIII in the

culture medium upon stimulation with PMA (Figure 4B). Our results show that FVIII is released from WPBs upon stimulation with PMA. However, in quantitative terms, release from this subcellular compartment is limited as opposed to non-stimulated release. It has recently been shown that in absence of a stimulus, so called 'basal release' of WPBs accounts for the majority of VWF released by endothelial cells.[32] We investigated whether the rapid accumulation of FVIII in the conditioned medium of transduced BOECs can also be attributed to basal release of WPBs containing FVIII. Transduced BOECs were incubated with BFA which prevents constitutive vesicular transport from the *trans*-Golgi network.[32] Treatment with BFA does not deform pre-formed WPBs and therefore has no effect on basal and regulated secretion.[36] In accordance with data obtained in HUVEC[32], BFA only partly blocks release of VWF into conditioned medium in BOECs (Figure 5A). In contrast, release of FVIII is almost completely abolished in the presence of BFA (Figure 5B). These findings suggest that, in contrast to VWF, the majority of synthesized FVIII is released in a constitutive manner and not via basal release of WPBs.

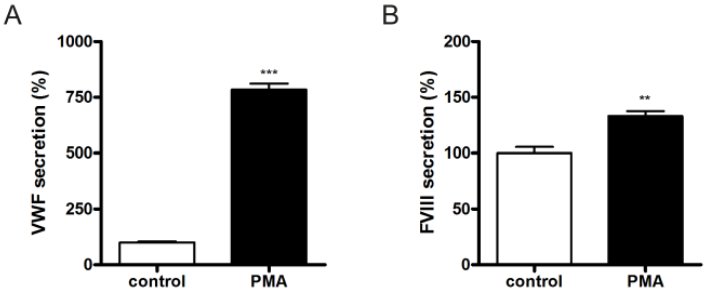

Figure 4. Regulated release of FVIII and VWF from PMA-stimulated BOECs. FVIII-transduced BOECs were stimulated for one hour with 50 ng/mL PMA. (A) VWF antigen and FVIII antigen (B) in the conditioned medium was quantified by ELISA. Values represent the mean ± SD of three experiments. **p<0.01, ***p<0.001.

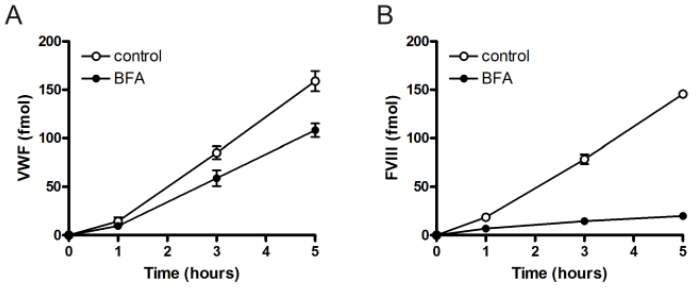

Figure 5. Quantitative analysis of the secretion pathways of FVIII and VWF in FVIII-transduced blood outgrowth endothelial cells. Release of FVIII from FVIII-transduced BOECs was analyzed over a 5-hour period in the presence of 5 µM BFA. (A) VWF antigen in the conditioned medium was quantified by ELISA. Values represent the mean±sd of three experiments. Open circles represent controls, closed circles represent secretion of VWF in the presence of BFA. (B) FVIII antigen in the conditioned medium was quantified by ELISA. Values represent the mean±sd of three experiments. Open circles represent controls, closed circles represent secretion of FVIII in the presence of BFA.

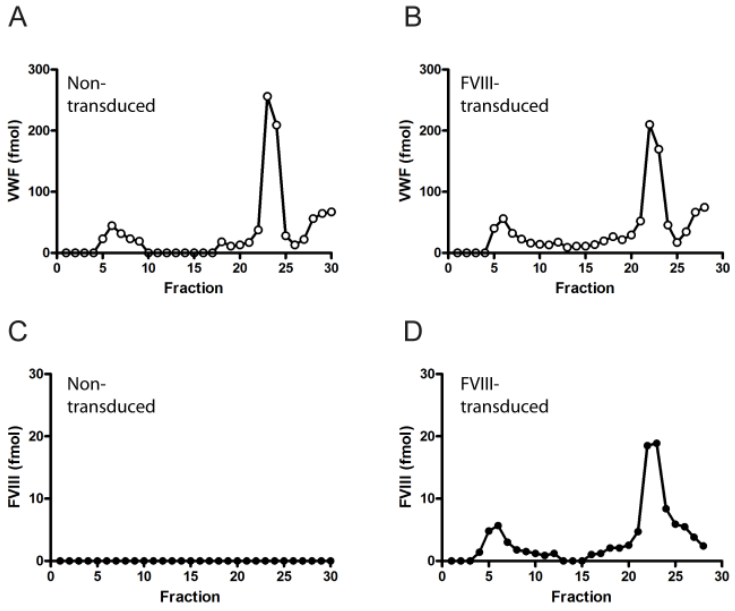

Figure 6. Subcellular fractionation and density gradient analysis of FVIII-transduced BOECs. Subcellular fractionation was performed on non-transduced BOECs (A,C) and FVIII-transduced BOECs (B,D). VWF antigen (A,B) and FVIII antigen (C,D) in the various fractions were quantified by ELISA (A,B). The first peak, fractions 4-10, corresponds to the dense WPB fraction, whereas the second peak, fractions 20-25, contains ER, Golgi apparatus and constitutively released vesicles. Shown is a representative experiment of two independent fractionations.

Quantitative determination of the amount of VWF and FVIII stored in Weibel-Palade bodies

These findings indicate that part of the synthesized FVIII is stored in WPBs whereas the remainder is secreted via the constitutive pathway. To assess this in a quantitative manner, we determined the amount of FVIII that is stored in WPBs. Homogenates of BOECs were subjected to density gradient centrifugation and the amount of VWF and FVIII was determined in the various fractions by ELISA. A representative fractionation is shown in Figure 6. The first peak (fractions 4-10) corresponds to the high-density WPB-containing fractions, whereas the second peak (fractions 20-25) contains the subcellular fractions derived of organelles of the secretory pathway (endoplasmatic reticulum (ER), Golgi, *trans*-Golgi network) and constitutively released vesicles.[37] The amount of VWF stored in WPBs was similar for non-transduced (12-15%; Figure 6A) and FVIII-transduced (15-21%; Figure 6B) BOECs. The percentage of FVIII stored in WPBs in FVIII-transduced BOECs was similar to the percentage of VWF stored (15-22%; Figure 6D). The average molar ratio of FVIII to VWF in the WPBs was approximately 1:15. These findings demonstrate that a significant portion of synthesized FVIII is co-targeted to WPBs in transduced BOECs.

Discussion

Blood outgrowth endothelial cells provide a cellular delivery device for FVIII that also produces its natural carrier protein VWF. In this study, we analyzed the potential of WPBs to serve as a releasable storage compartment for VWF and FVIII, which may have advantages with respect to high-level, on-demand secretion of preformed FVIII-VWF complexes that display a prolonged half-life. It has been well-established that the half-life of FVIII is reduced in patients with severe von Willebrand disease.[38] VWF protects FVIII from proteolytic degradation in the circulation and also interferes with the binding of FVIII to its clearance receptors.[39,40] Recently, VWF has also been shown to inhibit the uptake of FVIII by antigen presenting cells thereby providing a possible modulating effect on the development of an immune response to infused FVIII in patients with hemophilia A.[41] In addition, studies in megakaryocytes have suggested that co-release of VWF and FVIII may protect FVIII from inhibitory antibodies.[42,43]

We demonstrate that BOECs respond robustly to various stimuli with an increase in the amount of VWF secreted and a reduction in the number of WPBs (Figure 1). We therefore conclude that BOECs do not differ from HUVEC in terms of secretagogue responsiveness and indeed contain a recruitable WPB storage pool. Expression of FVIII in BOECs resulted in storage of FVIII in virtually all WPBs (Figure 3A). These vesicles were capable of recruiting the trans-membrane protein P-selectin (Figure 3A), which identifies these FVIII-containing organelles as true WPBs. FVIII- containing WPBs within transduced BOECs display round, spherical structures, which differ in morphology from the characteristic, elongated structures observed in the absence of FVIII (Figure 3B). Remarkably, in a previous report in which canine FVIII was expressed in canine BOECs, FVIII did not co-localize with VWF in WPBs.[10] This observation suggests that canine FVIII differs from human FVIII in its ability to co-target to WPBs. Alternatively, the amount of canine FVIII stored within WPBs may be too low to allow for detection by indirect staining with polyclonal anti-porcine FVIII antibodies. We have previously observed that the use of intrinsically labeled FVIII-GFP provides a superior means to address the subcellular localization of synthesized FVIII when compared to indirect staining using monoclonal or polyclonal antibodies.[20]

Quantitative assessment of FVIII storage using density gradients revealed that approximately 20% of total intracellular FVIII is present within WPBs (Figure 6D). Nevertheless, the amount of FVIII which can be released upon regulated secretion is limited compared to non-stimulated secretion (Figure 4B). This finding shows that the majority of synthesized FVIII is released independent of the presence of WPB secretagogues. Non-stimulated secretion may result from release through the constitutive pathway or from basal secretion of storage organelles that are released in a spontaneous fashion. Here, we show that the majority of FVIII is released in a constitutive manner and that release of FVIII does not result from rapid turnover of WPBs in the absence of a stimulus (Figure 5B).

Although the beneficial effect of VWF expression in BOECs in terms of FVIII storage remains to be established, we have demonstrated that lentiviral transduction of BOECs with FVIII results in high expression levels of FVIII (1.6±1.0 pmol/1x10^6 cells/24 h) that persist during

>30 days of culture. In fact, expression levels are higher than those obtained in established cell lines. Expression levels were 30-fold higher than those reported in a study in which BOECs were transfected using a non-viral transfection method[22] and similar to studies in which canine BOECs were transduced using a lentiviral vector encoding canine B-domain deleted FVIII or cord blood-derived endothelial progenitor cells (CBECs) using a lentiviral vector encoding human B-domain deleted FVIII.[10,34] Various studies have shown that endothelial cells are capable of secreting high levels of bio-active, heterodimeric FVIII. Therefore, endothelial cells seem to be a particularly suitable delivery device for *ex vivo* gene therapy for hemophilia A.

It is conceivable that constitutively released FVIII is cleared from the circulation more rapidly compared to the FVIII which is secreted together with VWF via regulated secretion of WPBs. In order to further benefit from co-storage of a FVIII-VWF inducible pool, it may be necessary to enhance the targeting efficiency of FVIII to WPBs. As targeting of FVIII to WPBs is independent of high-affinity interaction between FVIII and VWF[20,35], we suggest that attempts should be made to increase the amount of VWF stored in WPBs. One approach may be to co-transduce BOECs with VWF. De Meyer *et al*.[44] have recently demonstrated that WPB formation in VWD type 3 BOECs is restored upon lentiviral transduction with VWF. Another potential approach may be to over-express the transcription factor KLF2 which has recently been shown to increase the average number of WPBs in HUVEC.[45]

Authorship and Disclosures

MvdB and EAMB performed research, analyzed data and wrote the paper; RPH and NAK contributed essential protocols, techniques and/or materials; JV and KM conceived and designed research, analyzed data and wrote the paper. The authors reported no potential conflicts of interest.

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Chapter 3

Targeting of factor VIII variants with impaired von Willebrand factor binding to Weibel-Palade Bodies in endothelial cells

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Abstract

Point mutations resulting in reduced factor VIII (FVIII) binding to von Willebrand factor (VWF) are an important cause of mild/moderate hemophilia A. Treatment includes desmopressin infusion, which concomitantly increases VWF and FVIII plasma levels, apparently from storage pools containing both proteins. The source of these FVIII-VWF co-storage pools and the mechanism of granule biogenesis are not fully understood. We studied intracellular trafficking of FVIII variants implicated in mild/moderate hemophilia A together with VWF in HEK293 cells and primary endothelial cells. The role of VWF binding was addressed using FVIII variants displaying reduced VWF interaction. Binding studies using purified FVIII proteins revealed moderate (R2150H, Del2201, P2300S) to severe (Y1680F, S2119Y) VWF binding defects. Expression studies in HEK293 cells and primary endothelial cells revealed that all FVIII variants were present within VWF-containing organelles. Quantitative studies showed that the relative amount of FVIII storage was independent of various mutations. Substantial amounts of FVIII variants are co-stored in VWF-containing storage organelles, most likely by virtue of their ability to interact with VWF at low pH. Our data suggest that the potential of FVIII co-storage with VWF is not affected in mild/moderate hemophilia A caused by reduced FVIII-VWF interaction in the circulation. These data support the hypothesis that Weibel-Palade bodies comprise the desmopressin-releasable FVIII storage pool *in vivo*.

Introduction

Factor VIII (FVIII) is an essential cofactor in the factor Xa (FXa) generating complex by accelerating the factor IXa (FIXa) mediated-conversion of factor X (FX) into activated factor X (FXa).[1] The FVIII translation product consists of a 19 amino acid signal peptide followed by a 2332 amino acid precursor protein that is organized in a distinct domain structure: A1-a1-A2-a2-B-a3-A3-C1-C2. Due to intracellular proteolytic processing, FVIII circulates in plasma as a heterodimer consisting of a 90-220 kDa heavy chain (A1-a1-A2-a2-B) and a 80 kDa light chain (a3-A3-C1-C2). The heavy chain and light chain of FVIII remain associated through a variety of interactions, some of which are metal-ion dependent.[2] In the circulation, FVIII travels in complex with its carrier protein von Willebrand factor (VWF), preventing premature clearance and proteolytic degradation of FVIII.[1]

A defect in the gene encoding for FVIII results in the X-chromosome linked bleeding disorder hemophilia A. Large deletions, frame-shifts, premature stop codons or intron inversions are most commonly associated with severe hemophilia A and result in functional FVIII levels below 1%.[3] Severe hemophilia A patients are treated with on-demand or prophylactic protein replacement therapy using plasma derived or recombinant FVIII concentrates. Point mutations and small in-frame insertions or deletions in the FVIII gene generally result in a moderate or mild hemophilia A phenotype with circulating functional FVIII plasma levels between 1-5% and 5-30% respectively.[3] The molecular mechanisms that underlie moderate and mild hemophilia A include defects with respect to biosynthesis, altered interaction with factor IXa, reduced binding to phospholipid membranes, impaired thrombin activation, impaired stability in the circulation or a reduced ability to associate with VWF in plasma.[3,4] In addition to protein replacement therapy, mild or moderate hemophilia A patients can be treated with infusions of the vasopressin analogue desmopressin (DDAVP).[5,6]

Administration of DDAVP releases both VWF and FVIII in the circulation.[7] The source of DDAVP-releasable VWF and FVIII has not been established. However, several lines of evidence suggest that FVIII and VWF are synthesized and stored within the same cell.[8,9] This view is supported by the observation that *in vitro* co-expression of VWF and FVIII results in storage of FVIII in VWF-containing organelles.[10-15] Lung microvascular endothelial cells and liver sinusoidal endothelial cells both synthesize VWF and FVIII *in vivo*.[16-18] Recently, it has been shown that endothelial cells from several vascular beds, including the hepatic sinusoid and pulmonary vascular circulation, can synthesize and secrete FVIII.[19]

We have previously demonstrated that FVIII trafficking to VWF-containing storage organelles is independent of high-affinity binding to VWF.[13,15] VWF 2N variants that do not bind FVIII are still able to induce FVIII storage in WPBs[15], providing a rationale for the observed DDAVP-induced release of FVIII and VWF in type 2N VWD patients.[20] It remains unknown whether, in addition to type 2N VWD patients, co-storage of FVIII and VWF may also underlie the DDAVP-mediated increase of FVIII plasma levels in patients suffering from mild/moderate hemophilia. The aim of this study was therefore to analyze VWF co-storage for a panel of FVIII variants associated with mild/moderate hemophilia A due to reduced binding to VWF. For

these studies, we selected 5 FVIII variants that have been established to cause mild to moderate hemophilia A due to reduced binding to VWF.[21-24] We analyzed targeting of these FVIII variants to VWF-containing granules in heterologous HEK293 cells as well as in primary endothelial cells. Our results demonstrate that, despite impaired complex assembly with VWF, all FVIII variants retain their capability to traffic to VWF-containing organelles. These data support the hypothesis that FVIII-containing WPBs represent a desmopressin-releasable storage pool of VWF and FVIII *in vivo*.

Materials and methods

Plasmid mutagenesis

Construction of normal VWF[13], VWF-CFP with the Cyan Fluorescent Protein moiety replacing the A2 domain and VWF-CFP R763G[13], B-domain deleted (del746-1639) FVIII-YFP with the Yellow Fluorescent Protein moiety replacing the B-domain (FVIII-YFP)[13] and P-selectin[25] in pcDNA3.1(+) have been described previously. Point mutations and deletions in FVIII-YFP were introduced by Quick Change mutagenesis™ (Supplemental Table 1). B-domain deleted FVIII variants lacking the YFP moiety (FVIIIdB) were created by removal of the YFP moiety by Quick Change mutagenesis™ (Supplemental Table 1). FVIIIYFP variants and B-domain deleted FVIII variants were ligated into the lentiviral self-inactivating vector under control of the CAG promoter consisting of the chicken β-actin promoter, CMV enhancers and a large synthetic intron[14] using *NheI* and *NotI*.

Expression and purification of recombinant proteins

HEK293 cells were grown in DMEM-F12 medium supplemented with 10% FCS, 100 U/ml penicillin and 100 mg/ml streptomycin (BioWhittacker, Verviers, Belgium). HEK293 cell-lines, stably expressing recombinant protein were produced as described.[26] Recombinant VWF containing high molecular weight multimers and FVIII variants were purified and analyzed as described.[13]

Binding of FVIII-YFP variants to VWF

Pseudo-equilibrium binding of FVIII variants to VWF using an ELISA-based format was performed and analyzed as described.[13] Association and dissociation of FVIII variants to normal VWF was assessed by Surface Plasmon Resonance (SPR) analysis employing a BIAcore 3000 biosensor (Biacore AB, Uppsala, Sweden).[15] Recombinant normal VWF, VWF-CFP or VWF-CFP R763G (22 fmol/mm^2) was immobilized onto a CM5 sensor chip using the amine coupling method according to the manufacturer's instructions. Binding to coated channels was corrected for binding in absence of VWF. Varying concentrations (1-15 nM) of FVIII-YFP variants were passed over the immobilized VWF in a buffer containing 150 mM NaCl, 5 mM $CaCl_2$, 5% (v/v) Glycerol, 0.005% (v/v) Tween-20 and

	Primers	
	Y1680F forward	5′-GATTTTGACATTTTTGATGAGGATG-3′
	Y1680F reverse	5′-CATCCTCATCAAAAATGTCAAAATC-3′
	S2119Y forward	5′-CAGACTTATCGAGGAAATTACACTGGAACCTTAATG-3′
	S2119Y reverse	5′-CATTAAGGTTCCAGTGTAATTTCCTCGATAAGTCTG-3′
	R2150H forward	5′-ATTATTGCTCGATACATCCATTTGCACCCAACTCAT-3′
	R2150H reverse	5′-ATGAGTTGGGTGCAAATGGATGTATCGAGCAATAAT-3′
	Del2201 forward	5′-ACCAATATGTTTACCTGGTCTCCTTCAAAAGCTCGA-3′
	Del2201 reverse	5′-TGAAGGAGACCAGGTAAACATATTGGTAAAGTAGGA-3′
	P2300S forward	5′-GTGAACTCTCTAGACCCATCGTTACTGACTCGCTAC-3′
	P2300S reverse	5′-GTAGCGAGTCAGTAACGATGGGTCTAGAGAGTTCAC-3′
	Deletion YFP forward	5′-AGAAGCTTCTCCCAGAATCCACCAGTCTTGAAACGC-3′
	Deletion YFP reverse	5′-GCGTTTCAAGACTGGTGGATTCTGGGAGAAGCTTCT-3′

Supplemental Table 1. QuickChange Mutagenesis primers.

20 mM Hepes (pH 7.4) at 25 °C with a flow rate of 20 μL/min. The sensor chip surface was regenerated using the same buffer containing 1 M NaCl. To study the influence of pH on the interaction between normal VWF and FVIII-YFP (Y1680F), FVIII-YFP (Y1680F) was passed over the immobilized normal VWF in buffers containing 150 mM NaCl, 5 mM $CaCl_2$, 0.005% (v/v) Tween-20 and 20 mM Hepes (pH 7.4 or 6.7) or 20 mM MES (pH 6.2 or 5.5).

Expression levels of FVIII-YFP variants

HEK293 cells stably expressing VWF-CFP were transfected using the calcium phosphate coprecipitation method essentially as described.[27] Briefly, 2.5×10^5 cells/10 cm^2 were seeded 24 hours before transfection on a gelatine-coated culture surface. Medium was refreshed 4 hours before transfection. Cells were transfected using 5 mg of plasmid DNA. Medium was refreshed 16 hours after transfection and cells were grown for an additional 72 hours. Production levels of FVIII variants were determined by chromogenic assay (Chromogenix, Milano, Italy). Amounts of FVIII light chain and heterodimer were determined by ELISA.[28]

Immunofluorescence analysis

HEK293 cells stably expressing VWF-CFP or FVIII-YFP Y1680F were transfected using the calcium phosphate co-precipitation method.[27] Blood outgrowth endothelial cells (BOECs) were isolated from 50 ml venous blood donated by healthy volunteers.[14] Lentiviral preparations of FVIII variants were produced and BOECs were transduced as described.[14] Cells were prepared for immunofluorescence as described before.[29] Rabbit polyclonal antibody anti-human CD62-P (BD PharMingen, San Diego, CA, USA) was used to visualize P-selectin and sheep polyclonal antibody TGN46 (Serotec, Oxford, United Kingdom) was used to stain the *trans*-Golgi network. VWF was stained using monoclonal antibody CLB-RAg20 and FVIIIdB (variants) were stained using FITC-labeled human monoclonal antibody EL14 IgG_4.[15] Alexa-594 and Alexa-633-conjugated secondary antibodies were from Invitrogen (Breda, the Netherlands). Confocal Laser Scanning Microscopy was performed using a Zeiss LSM510 (Carl Zeiss, Heidelberg, Germany). For immunohistochemical analysis results were analyzed using Zeiss LSM510 version 4.0 software (Carl Zeiss, Heidelberg, Germany) or Image J (freely available through http://rsbweb.nih.gov/ij/). To obtain 3-dimensional images, z-stacks of 0.4 mm sections of individual cells were acquired. A 3-dimensional reconstruction was created with Image Pro Plus 6.0 (Media Cybernetics, Breda, the Netherlands) and used to calculate the number of Weibel-Palade bodies within individual cells. In order to separate narrowly connected Weibel-Palade bodies, the 3D Watershed filter (threshold 10%) was applied. The number of Weibel-Palade bodies containing VWF and FVIII-VWF was quantified by using the volume measurements software in the 3D constructor module. Results were analyzed using GraphPad Prism 4 software.

Subcellular fractionation

HEK293 cells stably expressing VWF-CFP or normal VWF were transfected using 87.5 mg plasmid DNA per 175 cm^2 flask as described above. Per FVIII variant, two 175 cm^2 culture flasks were transfected. Subcellular fractionation using Percoll density gradient centrifugation was performed as described with minor modifications.[30] Briefly, cells were homogenized by 20 strokes in a ball-bearing homogenizer (Isobiotec, Heidelberg, Germany) with a 14 micron clearance. The homogenate was loaded on a Percoll gradient and centrifuged for 30 minutes at 100,000*g* and 4°C. Fractions (1.25 ml) were collected from the bottom and stored at -20°C. FVIII antigen was quantified by anti-light chain ELISA as described above. VWF antigen was quantified by ELISA essentially as described before.[31]

Results

Expression of FVIII variants in HEK293 cells

To address the role of amino acid substitutions that cause mild/moderate hemophilia A in the intracellular trafficking of FVIII, we have created 5 FVIII variants in B-domain deleted FVIII-YFP that have been established to cause mild to moderate hemophilia A due to reduced binding to VWF[21-24], including single Y1680F, S2119Y, R2150H, Del2201 or P2300S substitutions. The effect of the hemophilic replacements on secretion of FVIII was studied. FVIII-YFP variants were expressed in HEK293 cells stably expressing VWF-CFP and FVIII levels in conditioned medium were quantified based on chromogenic activity (Table 1). Transient expression of normal FVIII resulted in significant levels of FVIII in conditioned medium (4.±0.4 pmol FVIII activity/1×10^6 cells per 72 hours) (Table 1). Levels of FVIII variants carrying a Y1680F and S2119Y replacement or a deletion of A2201 were approximately 50% reduced whereas levels of FVIII variants carrying an R2150H or P2300S were more than 10-fold reduced (Table 1). The levels of VWF were not affected (6.7±0.8 pmol VWF/1×10^6 cells per 72 hours).

Purification of FVIII-YFP variants produced by HEK293 cells

To address the ability of the FVIII variants to associate with VWF, FVIII-YFP variants were purified from the conditioned medium of HEK293 cell-lines expressing FVIII-YFP variants (in absence of VWF) using immunoaffinity chromatography. Due to low production levels, the R2150H variant could only be purified partially. After purification, the values for the specific activity of all other variants were at least >2000 U/mg (Table 1). SDS-PAGE analysis demonstrated that all these FVIII variants were processed and secreted predominantly as a heterodimer with virtual absence of single chain FVIII (Figure 1A).

Pseudo-equilibrium FVIII/VWF binding

Binding of FVIII variants, including the partially purified R2150H variant, to VWF was analyzed under pseudo-equilibrium conditions (Figure 1B).[13] As described previously[13], FVIII readily

FVIII-YFP variant	Production levels pmol activity/ 1×10^6 cells/ 72 hours	Specific activity of purified variants units FVIII activity/ mg protein
Normal	4.1 ± 0.4	$3.2 \pm 0.3 \times 10^3$
Y1680F	2.3 ± 0.5	$2.1 \pm 0.2 \times 10^3$
S2119Y	2.0 ± 0.2	$3.6 \pm 0.3 \times 10^3$
R2150H	0.3 ± 0.1	$1.2 \pm 0.1 \times 10^3$
Del2201	2.0 ± 0.1	$2.1 \pm 0.1 \times 10^3$
P2300S	0.3 ± 0.1	$3.4 \pm 0.2 \times 10^3$

Table 1. Specific activity and production levels of FVIII variants. Production levels of FVIII were quantified by chromogenic assay. Specific activity of purified FVIII-YFP variants was evaluated as the FVIII activity (in U) per mg of protein. Each value represents the mean±sd of at least three measurements.

bound to VWF with high affinity in the subnanomolar range (Figure 1B). All FVIII variants demonstrated a binding defect, in the following qualitative ranking: R2150H>Del2201>P2300 S>Y1680F=S2119Y. While variants carrying a Y1680F or S2119Y replacement demonstrated a severe reduction in VWF binding, appreciable binding was observed for the variants carrying an R2150H and P2300S replacement or deletion of A2201 (Figure 1B).

Surface plasmon resonance analysis of FVIII/VWF binding

We further used Surface Plasmon resonance (SPR) analysis to study binding of individual FVIII to purified recombinant VWF containing high molecular weight multimers. The R2150H variant could not be analyzed as this variant was only partially purified. Representative SPR experiments

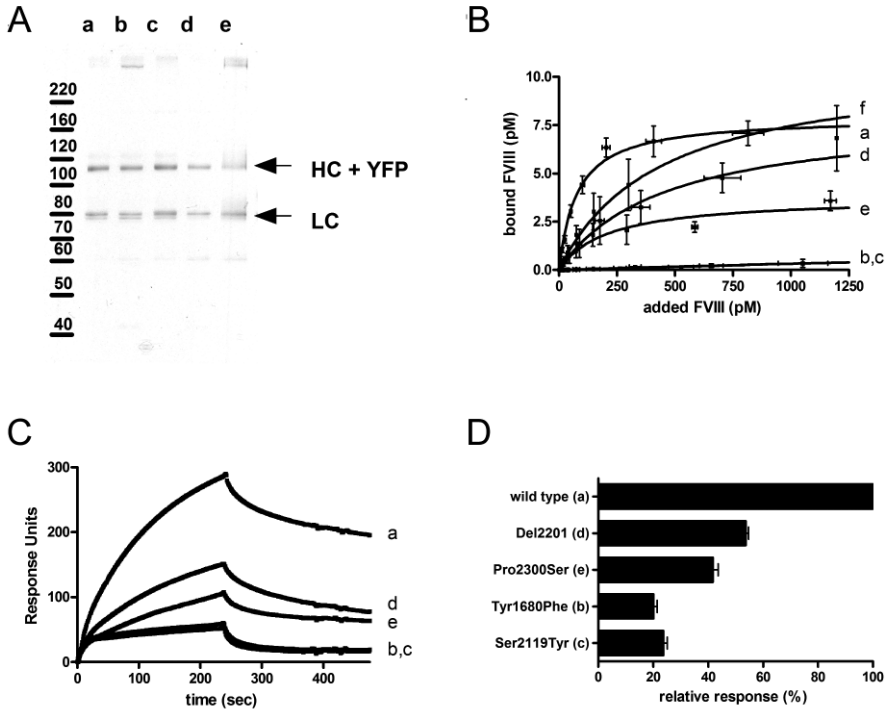

Figure 1. Binding of purified fluorescent FVIII variants to VWF. Letters represent normal FVIII (a), Y1680F (b), S2119Y (c), Del2201 (d), P2300S (e), and R2150H (f). (A) Purified protein preparations were analyzed by 7.5% SDS-PAGE under reducing conditions followed by silver staining. HC = heavy chain. LC = light chain. YFP = yellow fluorescent protein. (B) Pseudo-equilibrium binding of FVIII variants to VWF using an ELISA-based format was performed. The amount of FVIII bound to VWF was plotted against the amount of FVIII added. Data were analyzed by non-linear regression using a standard hyperbola. Each value represents the mean of three experiments. (C) SPR analysis of FVIII binding to VWF. FVIII variants were passed over a chip to which 22 fmol/mm^2 recombinant normal VWF was coupled in a buffer containing 150 mM NaCl, 5 mM $CaCl_2$, 5% (v/v) glycerol, 0.005% (v/v) Tween-20 and 20 mM Hepes (pH 7.4) at 25 °C with a flow rate of 20 ml/min. Association and dissociation phase were both followed for 240 seconds. Shown is the average curve of three injections of 2.5 nM of (each) FVIII variant. (D) Percentage of binding was calculated relative to normal FVIII after 235 seconds of association. Values represent the mean±SEM of at least 6 injections (1-10 nM FVIII variants).

are shown in Figure 1C and data are summarized in Figure 1D. SPR data demonstrated that normal FVIII readily bound to VWF (Figure 1C; curve a). All FVIII variants showed reduced binding to VWF (Figure 1C; curve b-e). Variants carrying a Y1680F and S2119Y replacement demonstrated a severe reduction in VWF binding. For these variants, some residual binding occurred which was rapidly lost during the dissociation phase (Figure 1C; curve b-c). Variants carrying a P2300S replacement or a deletion of A2201 demonstrated a binding defect that was less pronounced (Figure 1C; curve d-e). In addition, binding proved partially irreversible, presumably due to rapid rebinding of FVIII to the immobilized VWF immediately following dissociation. Rapid rebinding often implies that association is mass transport limited, which prohibits calculation of reliable binding kinetics.[32] Therefore, SPR data were used to qualitatively compare the different variants (Figure 1D). In agreement with the binding assay under pseudo-equilibrium conditions (Figure 1B), all FVIII variants demonstrated a binding defect, in the following qualitative ranking: Del2201>P2300S>Y1680F=S2119Y.

FVIII-VWF co-trafficking in HEK293 cells

We subsequently addressed the subcellular localization of FVIII variants in HEK293 cells. Normal FVIII staining is associated with the secretory pathway at the level of the *trans*-Golgi network and endoplasmatic reticulum.[13] Expression of hemophilic FVIII variants resulted in a similar localization (data not shown). As reported before [13], FVIII was observed in VWF-containing organelles in cells expressing VWF-CFP (Figure 2). Co-expression studies with P-selectin revealed that the FVIII/VWF-containing organelles retained the ability to recruit P-selectin. FVIII variants that demonstrate a moderate (Del2201) or severely reduced binding to VWF (Y1680F and S2119Y) were still able to co-traffic to VWF/P-selectin-containing granules (Figure 2). In addition, the FVIII variants carrying an R2150H or P2300S are stored in VWF/P-selectin-containing granules despite a moderate reduction in VWF binding and a more than 10-fold reduction in FVIII levels in conditioned medium (Figure 2).

Storage of FVIII variants in VWF-containing granules in HEK293 cells

So far, we have analyzed targeting of FVIII and VWF on a qualitative level. Our findings indicate that FVIII variants displaying a strongly reduced affinity for VWF can enter VWF-containing granules. We subsequently explored whether the amount of FVIII stored within VWF-containing granules depends on the affinity of FVIII for VWF. To address FVIII storage in VWF-containing granules in a more quantitative manner, we analyzed the amount of VWF-containing vesicles that also contained FVIII. We found that independent of the amino acid replacement, 80-100% of VWF-containing vesicles contained FVIII (Figure 3A). This observation indicates that targeting of FVIII variants to VWF-containing vesicles is independent of their affinity for VWF. In addition, we performed subcellular fractionations followed by density gradient centrifugation. Homogenates of transfected HEK293 cells were subjected to density gradient centrifugation and the amount of VWF and FVIII in the various fractions was determined by ELISA. Representative fractionations are shown in Figure 3B-G. The dense fraction containing the WPB-like organelles corresponds with fractions 4-9, whereas the second peak contains the

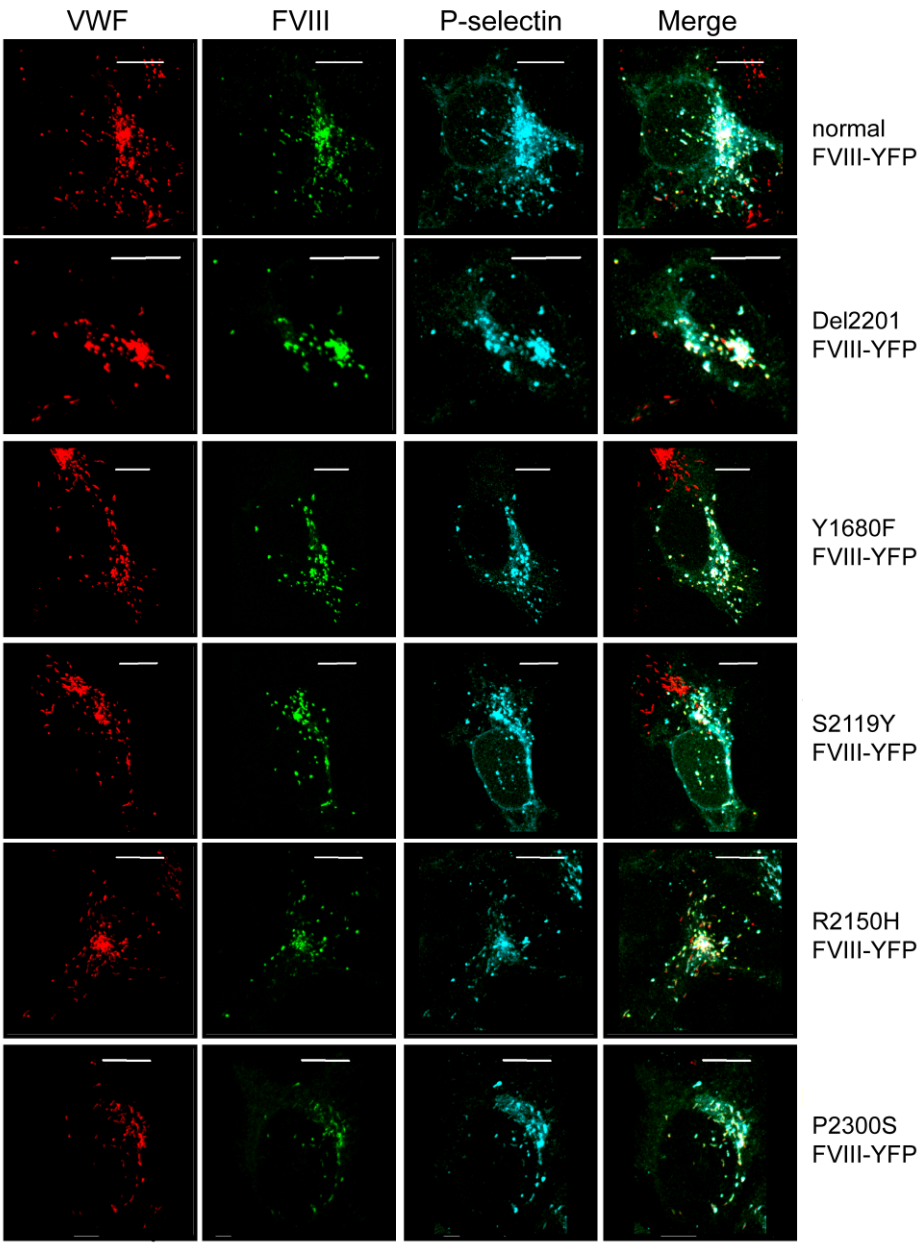

Figure 2. Intracellular localization of FVIII variants, P-selectin and fluorescent VWF. HEK293 cells stably expressing VWF-CFP were transfected with P-selectin and FVIII-YFP variants. Cells were stained for P-selectin using polyclonal anti-human CD62P antibody. VWF, FVIII and P-selectin are shown in red, green and blue respectively. Triple fluorescent detection is shown in the color merges. Shown are representative 3-dimensional projections of z-stacks (0.4 μm). The scale bar represents 10 μm.

subcellular fractions derived of organelles of the secretory pathway (ER, Golgi, *trans*-Golgi network) and constitutively released vesicles.[33] For normal FVIII, 21±6% of FVIII antigen and 16±5% of VWF antigen was found in the dense fraction of the cell (Table 2). To exclude that presence of the fluorescent YFP and CFP moieties influences targeting, subcellular fractionations were also performed using untagged FVIII and VWF variants. For untagged FVIII, 17% was targeted to the dense fraction of the cell containing normal VWF (data not shown). This indicates that the YFP/CFP moieties do not contribute to targeting of FVIII and VWF. Storage of all FVIII variants in VWF-containing granules was quantified. As expected, total intracellular FVIII antigen levels were reduced for the variants carrying an R2150H or P2300S replacement (Figure 3C-D). However, despite the reduced FVIII levels, a significant portion of the intracellular FVIII antigen was stored within the dense fraction of the cell. In addition, FVIII variants that display a moderate (Del2201) or severe reduction in VWF binding (Y1680F or S2119Y), are clearly stored in the fraction corresponding with VWF-containing granules (Figure 3E-G). These

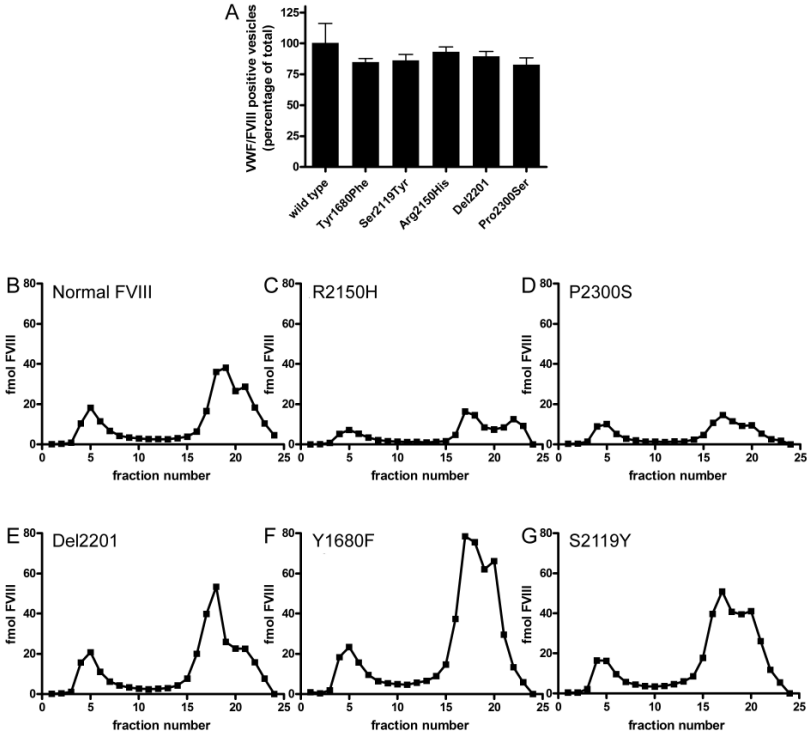

Figure 3. Quantitative analysis of storage of FVIII variants in VWF-containing organelles. HEK293 cells stably expressing VWF-CFP were transfected with FVIII-YFP variants. (A) Cells were analyzed by Confocal Laser Scanning Microscopy. Z-stacks of 0.4 μm sections of individual cells were acquired. A 3-dimensional reconstruction was created and the number of WPBs containing VWF and FVIII-VWF was quantified. Shown is the mean and standard deviation of at least 7 individual cells per FVIII variant. (B-G) Representative subcellular fractionations are shown. FVIII antigen was quantified by anti-FVIII light chain ELISA. The amount of FVIII light chain in fmol is plotted on the Y-axis against the fraction number on the X-axis.

FVIII-YFP variant	Percentage VWF Fractions 4-9	Percentage FVIII Fractions 4-9
Normal	16 ± 5	21 ± 6
Y1680F	16 ± 5	14 ± 6
S2119Y	19 ± 6	16 ± 4
R2150H	18 ± 4	16 ± 8
Del2201	16 ± 5	17 ± 4
P2300S	21 ± 4	18 ± 11

Table 2. Subcellular fractionation of transfected HEK293 cells. HEK293 cells stably expressing VWF-CFP were transfected with FVIII-YFP variants. FVIII antigen was quantified by anti-FVIII light chain ELISA. VWF antigen was quantified by ELISA. The amount of FVIII and VWF antigen in fractions 4-9 is divided by the total amount of FVIII and VWF antigen. The values represent the mean±sd of three measurements.

data demonstrate that substantial amounts of mild/moderate hemophilia A causing FVIII variants can be stored in VWF-containing storage organelles (Table 2).

FVIII-VWF co-trafficking in Blood Outgrowth Endothelial Cells (BOECs)

To validate the use of HEK293 cells as a model system, we also analyzed trafficking of FVIII variants in genuine endothelial cells. Previous studies have shown that BOEC provide an excellent model for studying the biosynthesis of VWF as well as targeting of FVIII to WPBs.[14,34] As was demonstrated for HEK293 cells, all FVIII variants were stored in organelles that also contained VWF. We conclude that FVIII trafficking to WPBs in BOECs is independent of the absence of high-affinity interaction with VWF (Figure 4A-B). To exclude the possibility that the YFP moiety contributes to FVIII targeting to WPBs, we also studied trafficking of Y1680F and S2119Y variants in a FVIIIdB background. Again, all FVIII variants were co-stored with VWF in WPBs (Figure 4C). Upon FVIII co-trafficking, WPBs lose their elongated shape and become spherical.[14,15] We have previously shown that this shape change is specific for the presence of FVIII as co-transfection of other WPB residents, including VWF and P-selectin, do not result in a shape change of the WPB.[15] Interestingly, the transition from elongated to spherical WPBs was observed for all FVIII variants, independent of their affinity for VWF and presence of the YFP moiety (Figure 4).

FVIII Y1680F is co-stored with the VWF type 2N variant VWF R763G

So far, our results show that targeting of FVIII variants to WPBs is not affected by a reduced binding of these variants to VWF. However, for most variants some residual binding to VWF is observed. To more precisely define whether FVIII binding to VWF is required for its targeting to WPBs, we used the previously described VWF type 2N variant (VWF R763G[15]) in conjunction with FVIII Y1680F. Despite a negligible interaction in our SPR experiments (data not shown), FVIII Y1680F was stored together with VWF R763G in HEK293 cells (Figure 5).

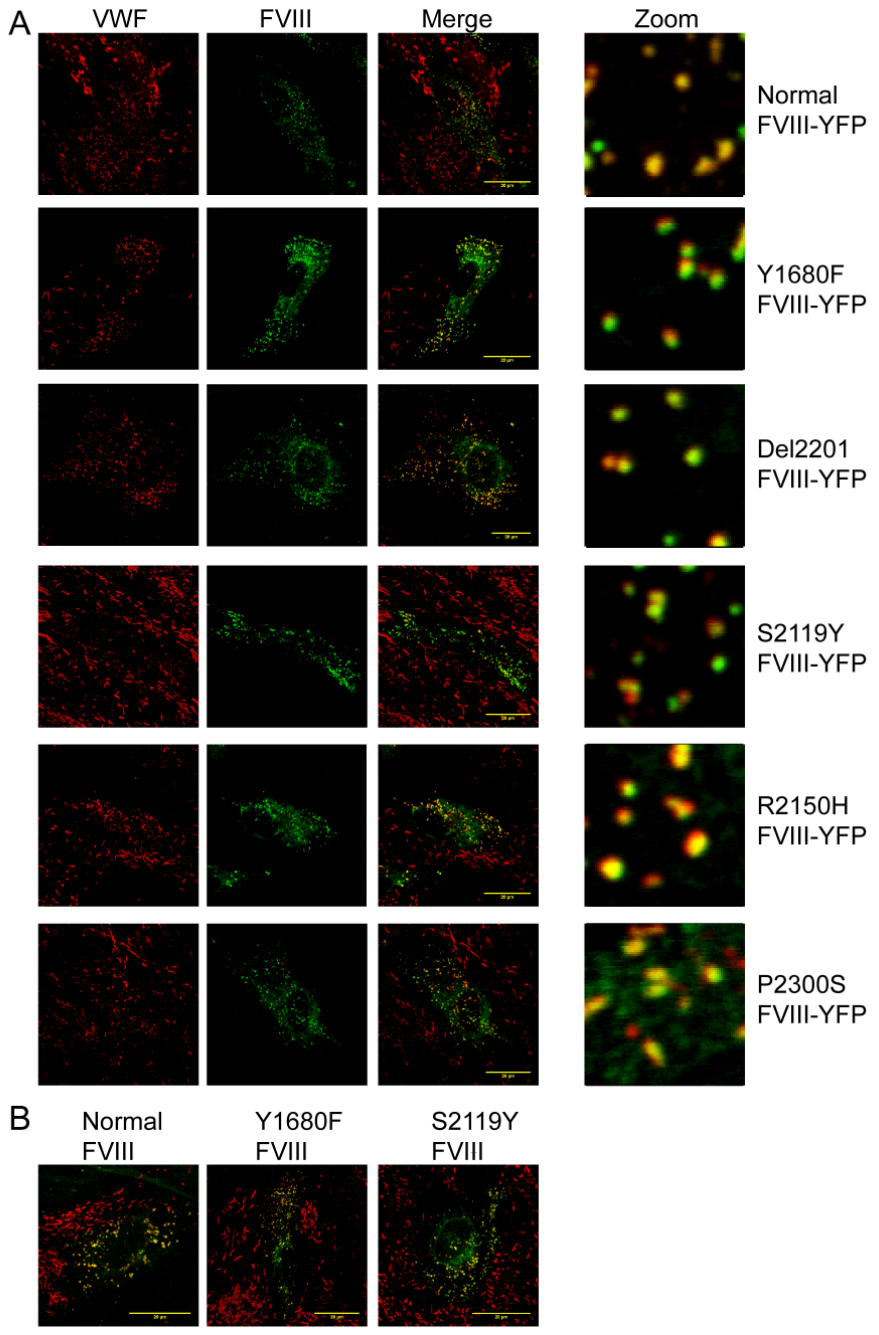

Figure 4. Co-trafficking of FVIII variants to Weibel-Palade bodies in endothelial cells. Human BOECs were transduced with lentiviral vectors encoding (A) FVIII-YFP, (B) FVIII-YFP variants carrying Y1680F, Del2201, S2119Y, R2150H and P2300S replacements or (C) Normal FVIIIdB and variants carrying Y1680F or S2119Y replacements. Cells were stained for VWF using monoclonal antibody CLBRAg20, shown in red (A,B,C) and for FVIIIdB (variants) (C) using FITC-labeled monoclonal antibody EL14 IgG_4. FVIII-YFP and FVIIIdB (variants) are shown in green. Double fluorescent detection is shown in the color merges. Shown are representative 3-dimensional projections of z-stacks (0.4 μm). The scale bar represents 20 μm.

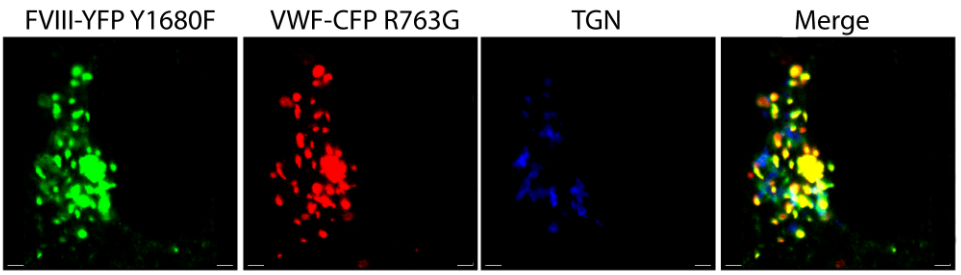

Figure 5. Co-storage of FVIII Y1680F with VWF type 2N variant VWF R763G. HEK293 cells stably expressing FVIII-YFP Y1680F were transfected with VWF-CFP R763G. Cells were stained for the trans-Golgi network using polyclonal antibody TGN46. Triple fluorescent detection is shown in the color merge. Shown is a representative 3- dimensional projection of z-stack (0.4 μm). The scale bar represents 10 μm.

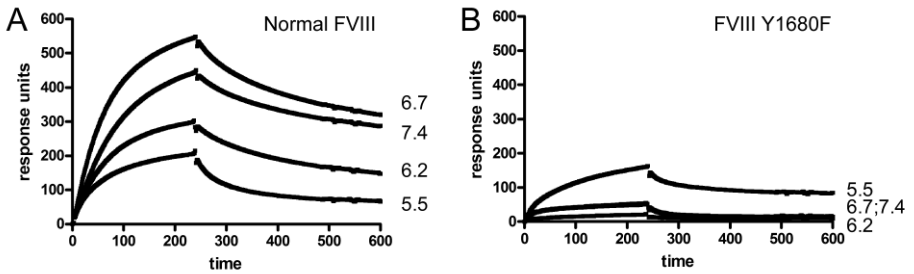

Figure 6. pH dependent binding of FVIII to VWF. 15 nM FVIII-YFP (A) or FVIII-YFP Y1680F (B) was passed over 22 fmol/mm^2 recombinant immobilized normal VWF in buffers containing 150 mM NaCl, 5 mM $CaCl_2$, 0.005% (v/v) Tween-20 and 20 mM Hepes (pH 7.4 and 6.7) or 20 mM MES (pH 6.2 and 5.5). Shown is a representative of 3 experiments.

Binding of FVIII to VWF is pH dependent

In the previous paragraphs we show that the impaired binding of FVIII variants to VWF does not abolish trafficking to WPBs. Sorting of proteins to storage organelles occurs at the levels of the *trans*-Golgi network (TGN). Acidification of vesicles along the secretory pathway provides the driving force of sorting of secretory hormones.[35] During formation of storage granules the intravesicular pH decreases from 6.2 to 5.5.[35] This prompted us to analyze the effect of pH on the interaction between VWF and FVIII. SPR binding experiments to VWF were performed for normal FVIII and the Y1680F variant in a pH ranging from 7.4 to 5.5. Binding of normal FVIII proved to be pH-dependent; at pH 5.5 reduced binding to VWF was observed (Figure 6A). Binding of FVIII Y1680F to VWF was severely reduced when compared to normal FVIII. No major differences were observed for this variant when binding studies were performed at a pH of 7.4, 6.7 or 6.2 (Figure 6B). Unexpectedly, binding of the Y1680F variant was increased at pH 5.5 and was similar to normal FVIII (Figure 6A-B). We therefore conclude that the pH optimum for VWF interaction differs between normal FVIII and FVIII Y1680F.

Discussion

Most mild and moderate hemophilia A patients can be effectively treated with DDAVP.[6] Treatment with DDAVP results in a concomitant increase of VWF and FVIII in the circulation, presumably by the release of storage pools that contain both FVIII and VWF. The formation of DDAVP-releasable co-storage pools requires endogenous synthesis of both FVIII and VWF. This is illustrated by the inability of DDAVP to mediate a rise in circulating FVIII levels in patients with severe (type 3) von Willebrand's disease or severe hemophilia A.[8,9] Restoring FVIII or VWF plasma levels to therapeutic levels by prophylactic protein replacement therapy, does not result in the formation of a DDAVP releasable co-storage pool in these patients.[8,9] Apparently, DDAVP-releasable storage pools cannot be restored by uptake of FVIII and VWF from the circulation.

While it is generally recognized that DDAVP releases VWF from WPBs, the origin and nature of the DDAVP-sensitive storage compartment of FVIII has not yet been defined. We and others have proposed that the DDAVP-induced rise of both FVIII and VWF argues for co-storage of both these proteins in WPBs. Pertinent to this point is our recent observation that VWF type 2N variants, despite a markedly decreased ability to bind to FVIII, drive co-trafficking of FVIII to VWF-containing granules.[15] In addition, we have previously demonstrated that the Y1680F FVIII variant is co-stored with VWF in WPBs despite its severely reduced interaction with VWF.[13] This raises the question as to whether VWF co-storage of FVIII variants displaying a reduced ability to associate with VWF represents a general phenomenon in mild/moderate hemophilia A. We have therefore extended our initial observation regarding the Y1680F variant to a larger panel of mild/moderate hemophilia A causing FVIII variants, including amino acid replacements in the FVIII C1 and C2 domains. In addition, we now have used a quantitative approach to assess trafficking of FVIII to VWF-containing granules in HEK293 cells. Moreover, we addressed co-trafficking of YFP-tagged as well as untagged FVIII variants in endothelial cells, with particular reference to the morphology of FVIII-containing WPBs.

We demonstrate that point mutations in the C1 and C2 domains of FVIII can have diverse effects on its synthesis, secretion and ability to bind to VWF without loss in cofactor function, in agreement with previously published data.[22] The ranking of VWF binding is the following: normal>R2150H>Del2201>P2300S>S2119Y=Y1680F (Figure 1). Remarkably, notwithstanding their reduced capacity to bind to VWF and/or reduced levels of synthesis, substantial amounts of moderate/mild hemophilia A causing FVIII variants can be stored in VWF-containing granules (Figures 2-4). Our quantitative studies showed that, independent of the amino acid replacement, relative amounts of FVIII stored in VWF-containing granules are similar (Figure 3). Assuming that co-expression and co-storage of FVIII and VWF does occur *in vivo*, our data provide a molecular explanation for the fact that hemophilia A patients suffering from impaired complex assembly of FVIII and VWF in the circulation can be effectively treated with DDAVP. In particular, hemophilia A patients carrying a Y1680F, S2119Y, R2150H replacement or deletion of A2201, respond to DDAVP treatment by a concomitant increase of FVIII and VWF.[21,36] In previous studies FVIII has been expressed in VWF-containing α-granules in mice. In

this model, the benefit of FVIII release concomitant with platelet activation was convincingly demonstrated.[37,38] Recently, targeting of FVIII to WPBs has been shown to restore hemostasis in a mouse model of hemophilia A.[39] This finding further emphasizes the hemostatic potential of DDAVP-induced FVIII release from WPBs.

The mechanism of FVIII-VWF co-trafficking to WPBs has not been elucidated so far. Even if we combine the FVIII Y1680F variant with a VWF type 2N variant that is severely defective in interacting with FVIII[15], co-storage with VWF still occurs (Figure 5). The observation that the FVIII Y1680F variant binds better to VWF under acidic conditions (Figure 6) suggests that pH dependent binding of FVIII and VWF along the secretory pathway may drive co-targeting of FVIII to WPBs. In cells that contain a regulated secretory pathway, the pH decreases from pH 7.4 in the ER, to pH 6.2 in the Golgi, to pH 5.5 in mature secretory granules.[35] This acidification process is essential for sorting and processing of regulated secretory hormones.[35] In endothelial cells, it has been postulated that the decreasing pH along the secretory pathway coordinates the disulfide-linked assembly of VWF multimers with their tubular packing.[40] VWF propeptide and D'-D3 dimers form helical tubules at pH 6.2 as a result of intersubunit disulfide bond formation.[40] Formation of helical tubules most likely underlies the formation of elongated WPBs.[41] It has been suggested that histidine residues in the propeptide function as pH sensors and control the disulfide bond formation within D'-D3.[42] Based on the pH-dependent binding of FVIII Y1680F to VWF (Figure 6), we suggest that 'histidine switch' may also control entry of FVIII in WPBs. Our data suggest that FVIII can bind VWF in multiple ways, including high-affinity interaction with a pH optimum of 6.7 and low-affinity interaction, independent of Y1680, with a pH optimum of 5.5. The mechanism of pH-dependent FVIII-VWF assembly therefore remains an intriguing question for further study.

Authorship and disclosures

MvdB performed experiments, analyzed data, made the figures, and wrote the paper; EAMB performed experiments, and made the figures; JV and KM designed research, analyzed data, and wrote the paper. The authors declare no competing financial interests.

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Chapter 4

Factor VIII alters tubular organization of von Willebrand factor stored in Weibel-Palade bodies

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Abstract

Weibel-Palade bodies (WPBs) are secretory granules that are exclusively present in vascular endothelial cells. During WPB biogenesis von Willebrand factor (VWF) multimers are packaged into tubules that direct elongation of these organelles. When WPBs release their content, VWF tubules unfurl and rapidly assemble into strings on the surface of endothelial cells onto which platelets adhere. By confocal microscopy we have previously observed a rounded morphology of WPBs in blood outgrowth endothelial cells (BOECs) transduced to express factor VIII (FVIII). Using correlative light-electron microscopy and tomography we now demonstrate that FVIII-containing WPB have disorganized, short VWF tubules. While normal FVIII and FVIII Y1680F interfered with formation of ultra-large molecular weight multimers, release of WPBs resulted in VWF strings of equal length as those from non-transduced BOECs. Although the length of the strings was unaffected, the strings released from both FVIII- and FVIII Y1680F-transduced BOECs had largely lost their ability to recruit platelets. These findings suggest that the intracellular FVIII-VWF interaction prevents the conformational change in VWF occurring after release from WPBs, which is needed to recruit platelets.

Introduction

In 1964 Weibel and Palade identified rod-shaped organelles containing fine tubules in endothelial cells of small arteries.[1] These organelles have a typical elongated shape of 100-200 nm in diameter and up to 5 mm in length, and are characterized by a uniform pattern of striations that runs along the longitudinal axis.[1] The structures became known as Weibel-Palade bodies (WPBs) and several years after their discovery, the multimeric glycoprotein von Willebrand factor (VWF) was identified as the major component of WPBs.[2] WPBs are secretory organelles specific for vascular endothelial cells that are released upon stimulation with agonists like thrombin and epinephrine.[3] The secreted VWF arrests bleeding by recruiting blood platelets to sites of vascular perturbation. Mutations in the gene encoding VWF are associated with the inherited bleeding disorder von Willebrand disease whereas increased levels of VWF are associated with venous thrombosis and adverse cardiac events in patients with preexisting cardiovascular disease.[4-6]

VWF is synthesized as a prepropolypeptide of 2813 residues that is cleaved into a propeptide (D1-D2) and a mature VWF monomer (D'-D3-A1-A2-A3-D4-B1-B2-B3-C1-C2-CK) of approximately 250 kDa.[5] VWF forms dimers in the ER through C-terminal disulfide bonds and multimerizes further in the Golgi through N-terminal disulfide linkage.[7-9] Multimers assemble into long, slightly twisted tubules that determine the typical cigar-shaped appearance of WPBs.[10-12] Formation of VWF tubules is initiated in the *trans*-Golgi network (TGN); further condensation of VWF tubules occurs in immature WPBs.[13] This process eventually results in paracrystalline packaging of VWF tubules[10] which strongly restricts the mobility of other WPB residents as demonstrated by a recent study employing fluorescence recovery after photobleaching.[14] The structural requirements for the pH- and Ca^{2+}-dependent formation of VWF tubules have recently been defined. Both propeptide and the amino-terminal D'-D3 domains are essential for *in vitro* formation of Weibel-Palade body like tubules.[11] Longer tubules are formed when the A1 domain is included suggesting that the A1 domain enhances tubule-formation by the amino terminal D1-D2-D'-D3 domains.[11] The physiological importance of the A1 domain in formation of VWF tubules is further suggested by formation of elongated pseudo-WPBs in HEK293 cells expressing a VWF variant truncated beyond the A1 domain.[15] Upon fusion of WPBs with the plasma membrane VWF tubules are exposed to physiological pH. This results in rapid disassembly of VWF tubules, which is presumably triggered by dissociation of the propeptide from D'-D3 dimers at neutral pH.[11,15] The newly released VWF multimers rapidly assemble into extended string-like structures on the surface of endothelial cells onto which platelets adhere.[15-17] The tubular organisation of VWF is thought to be essential for orderly secretion of these long VWF strings without tangling.[18]

Several groups reported recently that subsets of endothelial cells exist that synthesize FVIII and store it with VWF in WPBs.[19-22] Previously it was demonstrated that over-expression of FVIII in endothelial cells results in FVIII targeting to WPBs.[23-26] The targeting signal for FVIII sorting to WPBs is currently unclear, though the typical high-affinity interaction with VWF seems not required.[25,26] Interestingly, WPBs containing FVIII do not have their characteristic

elongated shape but appear as rounded vesicles that retain their ability to recruit P-selectin.[25,27] The mechanism behind this change in morphology is poorly understood and the effect of these alterations on functional properties of VWF is unknown. The aim of the present study was therefore to determine the intracellular effects of FVIII co-storage in WPBs on VWF, with particular reference to formation of VWF strings and platelet recruitment.

Materials and Methods

Materials

All chemicals used were of analytical grade. The μ-slide I 0.4 Luer ibiTreat flowchambers were from Ibidi (Munchen, Germany). Engraved glass-bottom dishes were from MatTek Coorporation (Ashland, MA, USA). Human serum albumin (CeAlb) was from Sanquin (Amsterdam, the Netherlands). RPMI1640, M199, DAPI and anti-GFP were obtained from Invitrogen (Breda, the Netherlands). DMEM-F12, EGM-2 bullet kit, penicillin and streptomycin were from Lonza (Walkersville, MD, USA). Collagen type 1 rat tail was from BD Biosciences (Uppsala, Sweden). Peroxidase-labeled polyclonal rabbit anti-human VWF antibody was from DAKO (Glostrup, Denmark). Tissue culture flasks, multidishes and microtiterplates (Maxisorp) were from Nunc (Roskilde, Denmark).

Transduction of Blood Outgrowth Endothelial Cells

Lentiviral vector pLV-CAG-FVIII-GFP has been described before.[24] B domain deleted FVIII-YFP with a Y1680F point mutation in pcDNA3.1(+) was constructed as described.[26] The LV-CAG-FVIII-YFP Y1680F plasmid was created by replacing fragment *NheI-XhoI* from pLV-CAG-FVIII-GFP for the corresponding fragment of the pcDNA3.1(+)FVIII-YFP Y1680F. Production of viral vectors, BOEC isolation and transduction with the viral vectors have all been described elsewhere.[24] FVIII-GFP transduced BOECs were ~80% positive for FVIII, FVIII-YFP transduced BOEC ~70% positive and FVIII-YFP Y1680F BOECs were ~45% positive. The latter were sorted by FACS to increase the percentage of positive cells. Confocal microscopy confirmed that almost all cells (~90%) were positive for FVIII Y1680F.

Correlative light and electron microscopy (CLEM) and electron tomography

BOECs expressing FVIII-GFP were grown on collagen-coated engraved glass-bottom petridishes (Mattek Corporation, USA) and CLEM was performed as previously described.[28] Briefly, confluent cells were fixed with 3.7% paraformaldehyde (PFA) in PBS for 30 minutes at 4°C and counterstained with DAPI. During fluorescence microscopy imaging, cells were kept in 3% PFA. Following imaging of nuclear staining and FVIII-GFP fluorescence with a Leica SP5 confocal microscope, the cells were fixed for electron microscopy with 2% glutaraldehyde in cacodylate buffer (0.1 M sodium cacodylate, pH 7.4) and processed for TEM as previously described[12]. Briefly, cells were post-fixed for 1 hour with 1% osmium tetroxide in cacodylate buffer and for 30 min in 1% tannic acid in cacodylate buffer. The samples were then dehydrated in ethanol (70–100%) and embedded in Epon using one beam capsule filled with Epon which was placed on the cell layer at the region of the engraved grid where the fluorescence imaging was performed. Epon was polymerized at 60°C overnight. To detach the capsule from the glass cover slip, the petridish was placed repeatedly on liquid nitrogen and hot plate. Thin sections (120 nm) were placed on one-slot grid coated with formvar and carbon, counterstained in 7% uranylacetate and Reynolds lead citrate and viewed with a Tecnai 12 transmission electron microscope at 120 kV equipped with a 4kx4k CCD camera (Model Eagle, Fei Company, The Netherlands) as described before.[28] After finding correlated structures, protein-A gold (10nm) was applied to the grids and used as fiducial marker for electron tomography. Double tilt series from -60° to +60° were collected at 1° interval using the Serial EM package.[29] 3D reconstructions were generated using the IMOD package.[30]

Immunogold labeling of cryo sections

FVIII-GFP transduced BOECs were fixed with 2% PFA and 0.2% glutaraldehyde in PHEM buffer (240 mM PIPES, 100 mM Hepes, 40 mM EGTA and 8 mM $MgCl_2$, pH6.9) for 2 hours at 4°C and stored in 0.5% PFA in PHEM buffer until processing for immunogold labeling as previously described.[31] Briefly, cells were scraped from the petridish, collected into an eppendorf tube and pelleted by gentle centrifugation. Thereafter the cell pellet was carefully resuspended into warm (37°C) gelatin and quickly pelleted. Gelatin was then let to set and harden on ice and small blocks of 1 mm^3 were cut with a razor blade and impregnated with 2.3 M sucrose in PBS overnight at 4°C. Thereafter the blocks were mounted on a pin using a drop of 2.3 M sucrose and snap frozen in liquid nitrogen. The blocks were stored in liquid nitrogen until sectioning. Cryo-sections of 90 nm were collected in a mixture (v/v) of 2.3M sucrose and 2% methyl-cellulose in double distilled H_2O, deposited on copper grids and stored at 4°C until labeling. Prior to labeling, the sections were warmed up on hard 1% gelatin at 37°C for 20 minutes. Grids were then deposited on a drop of PBS-"Gly" buffer (0.15 % glycine, 0.1 % BSA in PBS) to block non-specific binding four times for 1 minute. Thereafter the sections were labeled with a rabbit anti-GFP (Invitrogen) and indirectly with 15nm protein A-gold particles (provided by the laboratory of Dr. J. Klumperman, Utrecht Medical Center, The Netherlands) in PBS,1% BSA. After fixation in 1.5% glutaraldehyde in PBS to block the free rabbit antibody from the first incubation step, the sections were labeled with rabbit anti-VWF (DAKO), followed by incubation with 10 nm protein A-gold particles. Rinsing steps were performed in between using PBS-"Gly" buffer. The sections were contrasted and embedded in a mixture of uranylacetate-methylcellulose. As controls, anti-VWF or anti-GFP were omitted to rule out non-specific binding of protein-A gold. In addition, to rule out masking effects, the labeling was performed in reverse order, *i.e.* anti-VWF before anti-GFP. Acquisition of electron micrographs was performed on a Tecnai 12 transmission electron microscope mentioned above.

Von Willebrand factor multimer analysis

Conditioned medium from non-transduced, FVIII- and FVIII Y1680F-transduced BOEC was collected after 5 days of culture. Unstimulated cells were scraped and lysed in TBS, 1% Nonidet P40 supplemented with 10 mM benzamidine and one protease inhibitor cocktail tablet per 50 ml (Roche, Basel, Switzerland) at 4°C for 30 minutes. Lysate was spun down at 16,000*g* and 4°C for 5 minutes, after which the pellet was discarded. To measure regulated exocytosis of WPBs, BOECs were washed twice with PBS and stimulated with SF medium supplemented with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) for 1 hour. Collected medium was centrifuged for 10 minutes at 10,000*g*, supplemented with 10 mM benzamidine, and stored at -20°C until use. Human VWF antigen levels were determined by ELISA as described before.[24] Samples and a control of normal human plasma (NHP) were diluted in loading buffer (35 mM Tris, 2 mM EDTA, 9 M ureum, 1.2% (w/v) SDS, 0.1 % (w/v) bromophenol blue) to a final concentration of 62.5 ng/ml VWF. VWF multimers were analyzed by 2% (w/v) agarose gel electrophoresis followed by Western blot analysis using peroxidase-labeled polyclonal rabbit anti-human VWF antibody (DAKO). Plot profiles of blots were generated with ImageJ Software (Rasband, U.S. National Institutes of Health, Bethesda, MD, USA, http://rsb.info.nih.gov/ij/, 1997-2009).

Live cell imaging of flow experiments

BOECs were grown in collagen-coated flow chambers. EGM-20 medium (EGM-2 medium supplemented with EGM bullet kit and 20% FCS) was refreshed every other day until cells were confluent. SF medium supplemented with 100 μM histamine was perfused over the cells at 2.5 dynes/cm^2 for 10 minutes. SF medium containing

200×10^9 washed platelets/l was perfused over the cells at the same shear rate. Live-cell imaging was performed at 37°C on a confocal laser scanning microscopy using a Zeiss LSM510 equipped with Plan NeoFluar 40x/1.3 Oil objective (Carl Zeiss, Heidelberg, Germany). Images were taken using Zen 2009 LE software (Carl Zeiss, Heidelberg, Germany) at 10-second intervals for indicated time ranges after the onset of perfusion with histamine, unless indicated otherwise. For quantifying the length of the VWF strings and the number of platelets on the strings, tile scans were made of 1591×1591 mm. Images were processed and analyzed with ImageJ Software (Rasband, U.S. National Institutes of Health, Bethesda, MD, USA, http://rsb.info.nih.gov/ij/, 1997-2009). Number of platelets per mm VWF string and length of released strings was determined of at least 30 strings each. Differences between non-transduced and FVIII-transduced BOECs were evaluated by 2-tailed Student's t test using Graphpad Prism version 4.03 (Graphpad Software, San Diego, CA, USA). A value of $p<0.05$ was considered statistically significant.

Results

FVIII affects WPB morphology and VWF tubulation

The ability of VWF to assemble into helical tubules drives the elongation of endothelial cell specific WPBs. Previously, we have used confocal microscopy to show that FVIII containing WPBs display a rounded morphology.[24] We now employed electron microscopy to study the effect of FVIII on WPB morphology in more detail. Analysis of immunogold-labeled cryosections for VWF and GFP (to detect FVIII-GFP) showed that FVIII was found within large, rounded vesicles (Figure 1A). These structures were always labeled for VWF which confirms that the rounded structures were indeed WPBs that have become round. We also found structures that contained both VWF and FVIII which were neither rounded nor elongated but intermediate (Figure 1B). In contrast, elongated structures were only positive for VWF and represented genuine WPBs (Figure 1C). Moreover, we observed that FVIII was randomly distributed throughout WPBs in most cases and did not localize in specific areas in the WPBs, *e.g.* at the vesicle membrane. To quantify the size of FVIII-containing WPBs we measured the dimensions

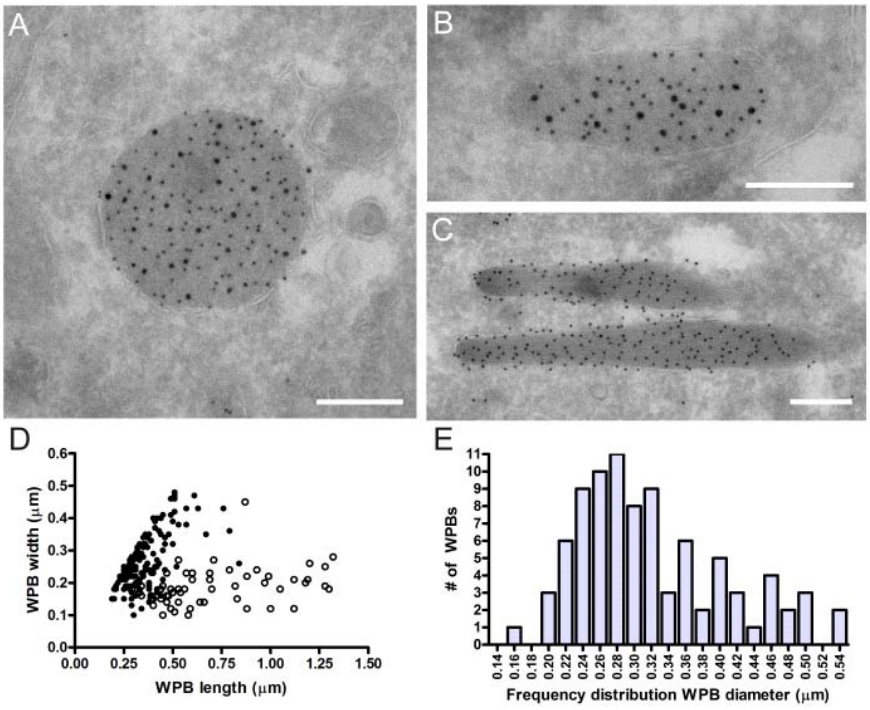

Figure 1. Rounded, electron-dense structures are positive for both FVIII and VWF. (A-C) TEM views of immunogold-labeled WPBs from FVIII-GFP transduced BOECs, double-labeled with anti-VWF (10 nm gold), and anti-GFP (15 nm gold) to detect FVIII-GFP. Scale bars represent 200 nm. (A) WPBs positive for VWF and FVIII mostly had a rounded morphology. (B) Intermediate, 'pear'-shaped WPB containing both FVIII and VWF. (C) Elongated WPBs are only positive for VWF. (D) Size distribution of 150 FVIII-positive (closed circles) and 56 FVIII-negative WPBs (open circles). Data includes both round and intermediate shaped FVIII-positive WPBs. (E) Diameter range of 88 selected FVIII-containing WPBs from D that were truly round.

of 150 randomly selected WPBs (Figure 1D). From this data set it appeared that the majority of the measured FVIII-containing WPBs had similar length and width ranging between 200 to 400 nm, suggesting a rounded shape. To determine the diameter range of rounded FVIII-containing WPBs, we first defined the dimensions of spherical WPBs using the criterion that a rounded WPB had a length of less than 1.5 times the width. Second, we only measured WPB whose membrane was visible to exclude measurements taken from WPBs that were sectioned at their far ends. Using these two criteria we found that the diameter range of rounded FVIII-containing WPBs is 170-540 nm with a median value of 300 nm (Figure 1E), which when considering the estimated section thickness of 90 nm and using the theoretical solution proposed by Parsons *et al*. gives a corrected value of 380 nm.[32] This was approximately 1.5-fold wider than a cross sectioned WPB that did not contain FVIII (100-450 nm with a corrected median of 230 nm). These findings confirm at the ultra-structural level that FVIII-containing WPBs display a rounded morphology.

The change in WPB morphology caused by storage of FVIII may indicate that VWF tubule formation is prohibited. We used correlative light-electron microscopy (CLEM) to determine whether VWF tubules are present in FVIII-containing WPBs. CLEM allows for identification of subcellular structures at EM level that were initially observed by fluorescence microscopy. In figure 2A three endothelial cells are displayed of which two express FVIII in rounded vesicles that are dispersed throughout the cells. Panel B provides an overlay of transmission electron micrograph and the fluorescence image displayed in panel A. In FVIII-negative cells, we could detect typical cigar-shaped WPBs containing long VWF tubules excluding the possibility of a defective VWF storage system in the cultured BOECs (Figure 2C-C'). Tubule-like structures were not visible in FVIII-containing WPBs visualized by two-dimensional TEM (Figure 2D'-D"). However, 3D electron tomography of FVIII-positive structures identified with CLEM revealed the presence of short and disorganized tubules (Figure 2E, 2F).

FVIII storage affects VWF multimerization

The lack of long VWF tubules in FVIII-containing WPBs suggests that FVIII interferes with expansion of VWF tubules. Previous findings have shown that the amino-terminal D1-D2-D'-D3 domains direct the formation of VWF tubules.[11,15] The same domains have also been implicated in formation of VWF multimers, while the D'-D3 region further contains the high-affinity FVIII binding site.[5] This raises the possibility that FVIII, by its association with VWF, interferes with the process of VWF multimerization. This issue was addressed by comparing VWF multimer patterns from non-transduced and FVIII-transduced BOECs. To assess the contribution of high-affinity FVIII-VWF interaction, we also examined BOECs transduced with FVIII carrying the Y1680F substitution. This variant effectively targets to WPB in HUVECs, despite its strongly reduced affinity for VWF.[26,33] Confocal microscopy revealed that FVIII Y1680F-containing WPBs displayed the typical rounded morphology (Chapter 3). As previously established, FVIII expression in BOECs did not appreciably affect VWF biosynthesis and regulated secretion.[24] Multimer patterns represent VWF released from FVIII-expressing cells, as approximately 70-90% of the cells were positive for FVIII.

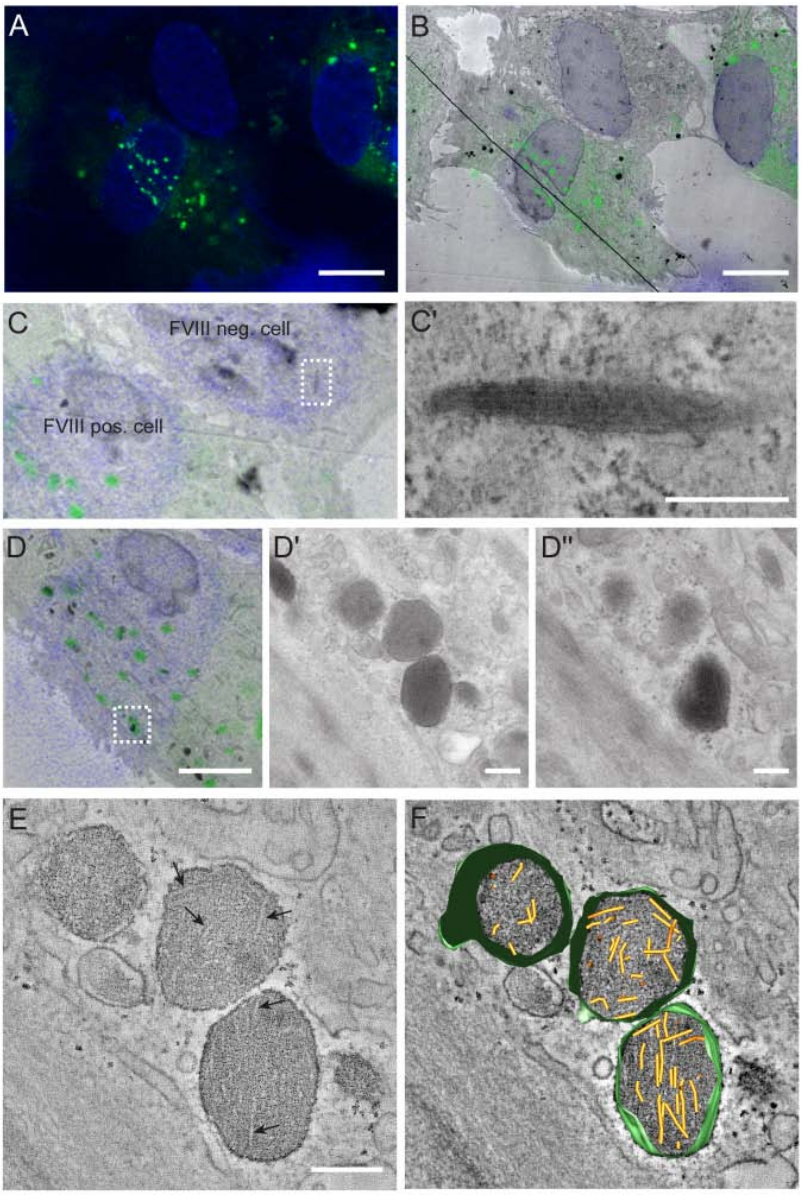

Figure 2. Correlation of FVIII-positive WPBs with rounded, electron-dense structures containing short VWF tubules. (A) Fluorescence image of BOEC expressing FVIII-GFP showing FVIII fluorescence in green and DAPI staining in blue. Scale bar 10 μm. (B) Overlay of transmission electron micrograph and the fluorescence image of the cells shown in A. The black line crossing the image represents a fold in the section. Scale bar 10 μm. (C) Zoom of B showing a FVIII negative and positive BOEC. (C') Higher magnification TEM view of the boxed area in C showing an elongated WPB that does not contain FVIII. Scale bar 250 nm (D) FVIII-positive structures correlating with FVIII-GFP fluorescence. Scale bar 4 μm (D'-D") Two consecutive sections of the structures boxed in overlay D. Scale bar 250 nm. (E) Digital slice through an electron tomogram of the electron-dense structures shown in D-D" showing FVIII-positive, electron-dense structures with disorganized, short VWF tubules. Arrows indicate longitudinal sections of VWF tubules. Scale bar represents 250 nm. (F) Three-dimensional model of tomogram shown in E. VWF tubules are indicated in yellow, green depicts vesicle membranes.

For non-transduced BOECs, agonist-induced WPB exocytosis resulted in release of VWF multimers containing substantially higher multimeric forms (Figure 3A) than found in cell lysate (Figure 3B). Apparently, BOEC-derived elongated WPBs are similar to those from HUVECs in that they contain the ultra-large high molecular weight (UL-HMW) multimers that are lacking in VWF from the constitutive secretion pathway.[8,34] In contrast, VWF from FVIII-containing, rounded WPBs was lacking the highest molecular weight forms (Figure 3A). Interestingly, the multimeric patterns from WPBs containing normal FVIII and FVIII Y1680F were indistinguishable (Figure 3A). This suggests that FVIII interferes with multimerization by a mechanism that does not require the high-affinity interaction involving sulphated tyrosine 1680. In cell lysates, however, the multimeric patterns from non-transduced and FVIII-transduced BOECs were more similar, although the VWF from non-transduced cells tended to display somewhat higher multimerisation (Figure 3B).

FVIII has recently been proposed to act as a cofactor for cleavage of VWF strings by ADAMTS13.[35] The multimer patterns of cell lysates (Figure 3B) do not reflect any apparent

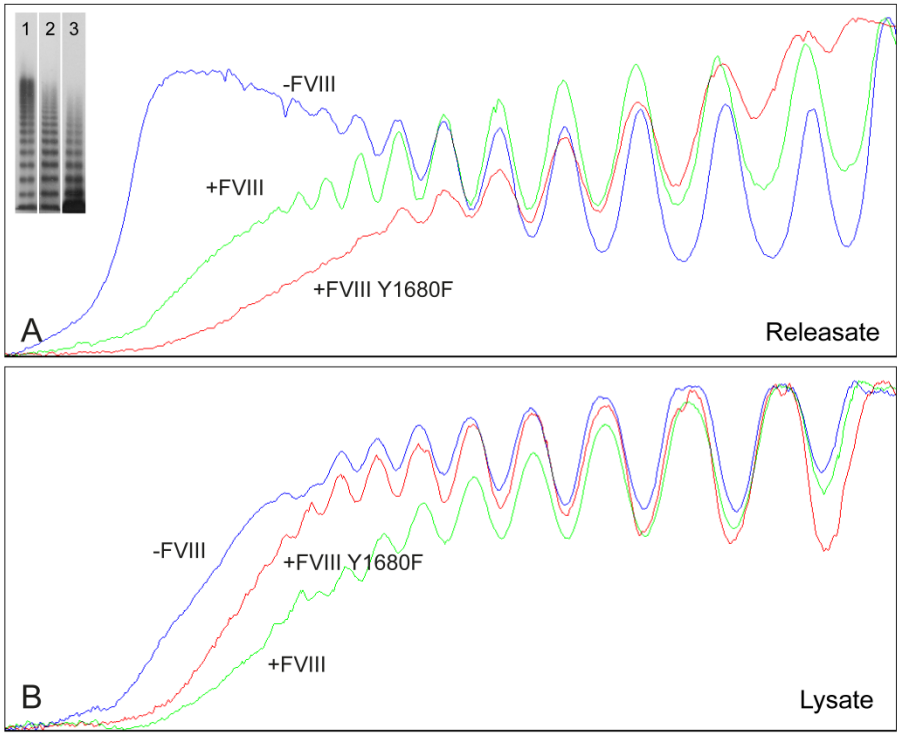

Figure 3. Multimer size of VWF secreted by non-transduced, FVIII- and FVIII Y1680F transduced BOECs. Plot profiles of VWF multimer patterns of releasate (A) and lysate (B) from non-transduced (1), normal FVIII- (2) and FVIII Y1680F (3) transduced BOECs. Multimers were analyzed by 2% agarose gel electrophoresis followed by Western Blot analysis using rabbit polyclonal anti-human VWF antibody. Plot profiles of blots were generated with ImageJ Software (Rasband, U.S. National Institutes of Health, Bethesda, MD, USA, http://rsb.info.nih.gov/ij/, 1997-2009).

ADAMTS13-mediated cleavage. Moreover, we were unable to detect any appreciable amounts of ADAMTS13 in BOECs by immunological methods (data not shown). Therefore, the absence of UL-HMW multimers in VWF secreted by FVIII-expressing cells is unlikely to be the result of increased cleavage by ADAMTS13 during exocytosis. We conclude that in rounded WPBs the UL-HMW multimers are lacking due to interference of FVIII in VWF multimerisation.

Rounded FVIII-containing WPBs release UL-VWF strings that bind fewer platelets

The absence of long VWF tubules in FVIII-containing WPBs raised the question whether FVIII-transduced BOEC could still release ultra-large (UL) VWF strings. UL-VWF strings play a key role in bleeding arrest as platelets adhere to released VWF strings, which ultimately leads to formation of a platelet plug. We examined the ability of FVIII-positive cells to release VWF strings by monitoring platelet adhesion to strings as described previously.[17,36] Following stimulation with histamine non-transduced BOECs released VWF strings that were readily visualized following addition of platelets (Figure 4A). Unexpectedly, VWF strings released from cells expressing FVIII were more difficult to detect as platelet binding was strongly reduced. Closer inspection revealed the presence of sparsely distributed platelets that were arranged in a linear fashion on the surface of FVIII-expressing BOECs (Figure 4B; see arrowheads).

The relation between FVIII expression and platelet recruitment to VWF strings was further explored using a quantitative approach. First, we determined the length of strings released by FVIII-expressing cells and compared this to the length of strings released from non-transduced BOECs. We found that, despite their shorter tubules, strings released by FVIII-positive cells were of similar length as VWF strings secreted by non-transduced BOECs, on average 338 mm and 360 mm, respectively (Figure 5A). Strings released from cells expressing FVIII Y1680F were slightly shorter (average of 297 mm), although this differences was not statistically significant. The length of VWF strings ranged from ~160-830 mm for non-

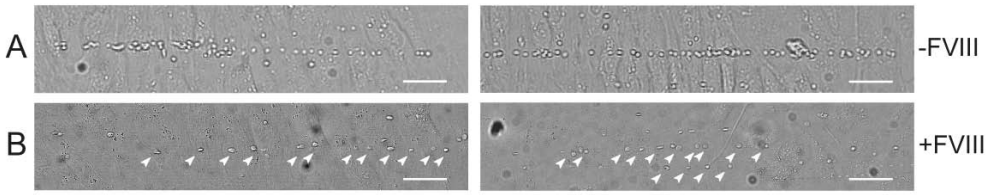

Figure 4. VWF strings released from FVIII-containing WPBs show reduced platelet binding properties. Two images of non-transduced (A) and FVIII-GFP transduced (B) BOEC stimulated with 100 μM histamine at 2.5 dynes/cm^2 for 10 minutes and subsequently perfused with washed platelets for 5 minutes. Platelet binding to VWF strings originating from non-transduced BOECs is more pronounced than platelet binding to strings released from FVIII-expressing BOECs. Scale bars represent 100 μm. Arrowheads indicate platelets bound to VWF strings released by FVIII-GFP transduced BOECs. Images were taken on a confocal laser scanning microscopy using a Zeiss LSM510 equipped with Plan NeoFluar 40x/1.3 Oil objective (Carl Zeiss, Heidelberg, Germany) at 37°C.

transduced BOECs, ~170-600 mm for FVIII-transduced, and ~125-930 mm for FVIII Y1680F-transduced BOECs. We then calculated the number of platelets bound per micrometer VWF string. Strings secreted by FVIII-expressing BOECs were able to bind an average number of 0.07 platelets per mm string, whereas normal VWF strings contained on average 0.18 platelets per mm string (Figure 5B). In other words, a typical string released from FVIII-positive cells of 338 mm can bind 24 platelets whereas regular strings recruit 65 platelets. Surprisingly, VWF strings released from FVIII Y1680F-positive cells displayed an intermediate defect in platelet binding as strings bound on average 0.11 platelets per mm string.

Discussion

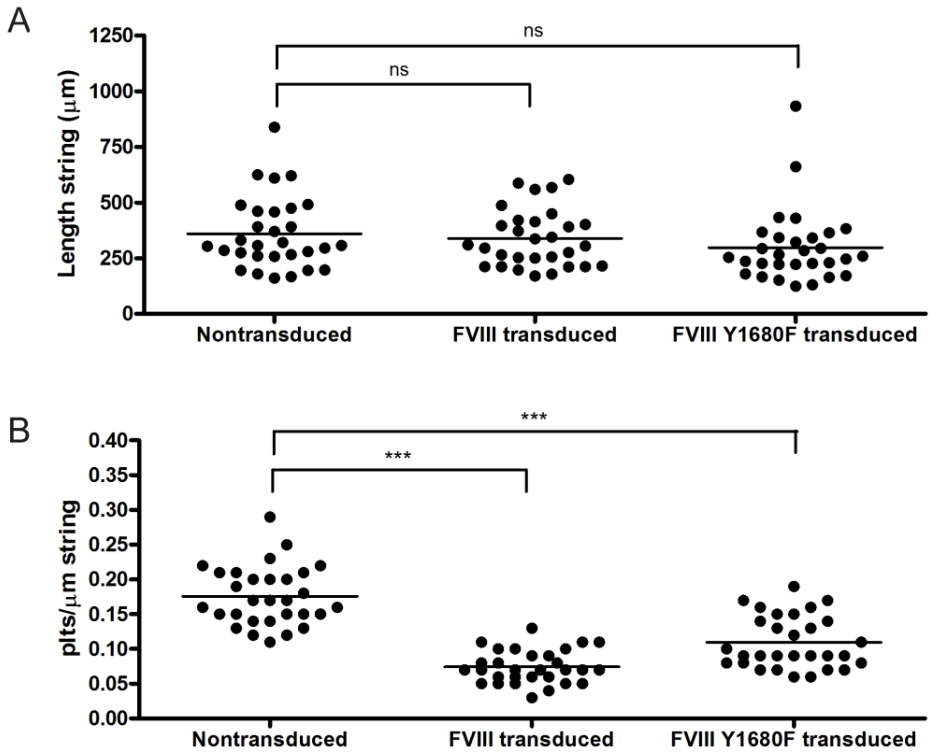

Figure 5. Quantification of platelet binding properties and length of VWF strings from FVIII- and FVIII Y1680F-transduced BOECs. Phase contrast tile scans were used to measure length of released VWF strings (A) and number of platelets per string (B) of at least 30 strings each from non-transduced, normal FVIII- and FVIII Y1680F transduced BOECs. (A) No significant differences were detected in the length of VWF strings released by non-transduced BOECs, FVIII-, and FVIII Y1680F-transduced BOEC, 360±29 μm, 338±23 μm (p=0.57) , and 297±30 μm (p=0.14), respectively. Values are given in mean±sd. (B) VWF strings released by FVIII- and FVIII Y1680F-transduced BOECs bind fewer platelets than strings released by non-transduced BOECs, 0.07±0.02, 0.11±0.04, and 0.18±0.04, respectively (mean±sd). *** p<0.001

While vascular endothelial cells in general are devoid of FVIII biosynthesis, recent evidence suggests the existence of specific subsets that do synthesize FVIII, and store FVIII together with VWF in WPB-like storage organelles.[19-22] The regulated FVIII secretion observed from these cells may represent the FVIII storage pool that is released *in vivo* upon administration of the vasopressin analogue DDAVP[22], and as such adds physiological significance to endothelial FVIII expression. In the present study, we have used BOECs to study the implications of FVIII expression in endothelial cells. We observed that FVIII-expression causes a variety of changes, including the size and organization of VWF tubules in WPBs (Figures 1-2), the size of the VWF multimers (Figure 3), and the recruitment of platelets to VWF strings once released from WPBs (Figures 4-5).

With regard to WBP morphology, it has been well established that VWF is stored in WPBs as helical tubules that provide these organelles with their typical elongated shape. Tubule formation occurs in the TGN where acidic pH and high Ca^{2+} concentrations promote binding of propeptide D1-D2 to the D'-D3 domains.[11] In the same compartment propeptide is cleaved from mature VWF, most likely by furin.[37,38] The propeptide remains non-covalently associated to D'-D3 domains thereby stabilizing the helical conformation of VWF tubules.[11,15] We observed that targeting of FVIII to WPBs disrupts the parallel alignment and compaction of VWF tubules into elongated WPBs (Figures 1 and 2). Possibly, FVIII may interfere with propeptide binding to the VWF D'-D3 domains, thereby preventing the extension of VWF tubules and the resulting elongation of WPBs. We have previously established that the molar ratio of FVIII to VWF in WPBs of FVIII-transduced BOECs is on average 1:15.[24] The relatively low amount of FVIII compared to VWF might explain why VWF tubulation is only partially impaired by FVIII. Our data seem compatible with a model in which FVIII blocks binding of propeptide during covalent addition of VWF multimers to the growing tubule. Interestingly, using a high-pressure freezing technique, Zenner *et al.* observed small structures resembling VWF tubules in cross-sections that were randomly distributed in membrane-bound rounded vesicles in the vicinity of the TGN.[13] They hypothesize that these structures represent so-called "tubular subunits" destined for fusion with other VWF tubules into large tubules. The lack of elongated WPBs in endothelial cells expressing FVIII thus may indicate that FVIII interferes with assembly of pre-tubules into long tubules.

Rounded WPBs have also been observed following over-expression of a VWF variant harboring an Y87S substitution defective in multimerization.[15,39] In addition, treatment of endothelial cells with agents that neutralize the acidic pH in secretory organelles, such as NH_4Cl, chloroquine and monensin, results in conversion of elongated to rounded WPBs.[15] While treatment with monensin induces loss of VWF tubules, monensin-treated endothelial cells are still able to release short VWF strings.[15] This is in agreement with our observation that, despite the presence of disorganized and short tubules, FVIII-containing WPBs are still able to release UL-VWF strings (Figures 4-5). Apparently, tubular organization of VWF is not essential for the formation of UL-VWF string on the surface of endothelial cells.

With regard to tubule length, Huang and coworkers reported that helical tubules created *in*

vitro from propeptide and D'-D3 dimers contain 4.2 dimers per turn with one helix segment spanning 11 nm.[11] Accordingly, a 5 mm measuring WPB with VWF tubules spanning the entire length has tubules containing ~3800 VWF subunits and a mass of ~975 million Da. This corresponds with an extended length of 250 mm, which is compatible with the length of a VWF string.[11,18] In our study (Figure 1), most FVIII-containing WPBs measure ~380 nm in diameter and do not contain tubules spanning the entire WPBs. In line with the above calculations these organelles should release strings no longer than ~19 mm. Our data show that FVIII-expressing endothelial cells release strings that are much longer, with an average length of 340 mm (Figure 5A). This argues in favor of linkage of VWF multimers post-release[40,41] or pooling of WPB content during multigranular exocytosis.[28]

Despite their normal length, VWF strings from FVIII-expressing cells display greatly reduced platelet recruitment (Figures 4 and 5B). In fact it was even more reduced than reported for monensin-treated cells, which completely lack VWF tubules.[15] This suggests that the reduction in platelet binding is not related to the decreased tubule length in these cells, but to the presence of FVIII. Theoretically, the reduction of platelet binding to VWF strings from FVIII-expressing cells may be due to steric hindrance, whereby FVIII shields part of the VWF A1 domain that is involved in platelet binding. Alternatively, the association with FVIII might interfere with the conformational changes in VWF that are needed for exposure of platelet binding sites in the VWF A1 domain.[5,42,43] We hypothesize that FVIII prevents this conformational change when it is bound to VWF strings prior to secretion and thereby reduces adhesion of platelets. Further studies are needed to establish whether endogenous, WPB-derived FVIII indeed interferes with formation of an 'active' conformation of the VWF A1 domain.

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Chapter 5

Factor VIII binds to von Willebrand factor strings released by endothelial cells

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Abstract

In endothelial cells von Willebrand factor (VWF) multimers are packaged into tubules that direct biogenesis of elongated Weibel-Palade bodies (WPBs). WPB release results in unfurling of VWF tubules and assembly into strings that serve to recruit platelets. Recently we have demonstrated that factor VIII (FVIII)-containing WPBs have short, disorganized VWF tubules that recruit fewer platelets. This implicates that FVIII and VWF associate inside WPBs. In this paper we studied whether or not WPB-derived FVIII is released in complex with VWF. We found that FVIII co-stored with VWF in WPBs remained associated with VWF on UL-VWF strings. Moreover, despite its decreased affinity for VWF, WPB-derived FVIII Y1680F also remained bound to VWF strings. These findings suggest that the FVIII-VWF interaction during WPB formation is independent of Y1680, and is maintained after WPB release on FVIII-covered VWF strings. While perfusion of histamine-treated BOECs with normal FVIII resulted in adequate binding of FVIII to VWF strings, exogenously added FVIII Y1680F bound VWF strings to a lesser extent. Apparently intracellular and extracellular assembly of FVIII-VWF complex involves distinct mechanisms, which differ with regard to the importance of Y1680-mediated interaction. Moreover, we demonstrate that VWF strings can bind FVIII from perfused FVIII-VWF mixtures containing physiological FVIII-VWF ratios. This raises the possibility that VWF strings may recruit FVIII from circulation to assist in local bleeding arrest.

Introduction

Weibel-Palade bodies (WPBs) are secretory organelles specific for vascular endothelial cells. These organelles are characterized by striations running along the longitudinal axis consisting of condensed von Willebrand factor (VWF).[1] VWF is synthesized as a prepropolypeptide that consist of a propeptide (D1-D2) and a mature VWF monomer (D'-D3-A1-A2-A3-D4-B1-B2-B3-C1-C2-CK).[2] VWF monomers undergo tail-to-tail dimerization through disulfide bond formation in the ER.[3] In the Golgi, VWF multimerizes by head-to-head disulfide linkage.[4,5] VWF multimers condense into long helical tubules that determine the elongated shape of WPBs.[6,7] Exocytosis of WPBs from endothelial cells releases VWF, which assembles into extended string-like structures known as ultra-large (UL-)VWF strings.[8,9] Platelets then adhere to these strings to assist in bleeding arrest.[10]

Besides recruiting platelet to sites of vascular damage, VWF functions as a molecular chaperone for factor VIII (FVIII), a co-factor for activated factor IX in the factor X-activating complex. It is thus far unclear where FVIII and VWF associate but complex formation with VWF significantly increases circulation time of FVIII.[11] The FVIII-VWF complex is sustained through interactions of the FVIII light chain (a3-A3-C1-C2) with the D'-D3 region on VWF. Proteolytic activation of FVIII removes the acidic a3 region (residues 1649-1689) that harbors a high-affinity VWF binding site.[12] Loss of a sulfated tyrosine at position 1680 in this a3 region results in impaired complex formation with VWF.[13]

FVIII is mostly synthesized in the liver although emerging evidence demonstrates that FVIII can also be produced in other tissues. For instance, while liver transplantation in hemophilic recipients can correct circulating FVIII levels, transplantation does not restore the FVIII pool released after desmopressin (DDAVP) treatment.[14] This indicates that there should be at least one additional extra-hepatic source for FVIII.[15] There is now increasing evidence to support the hypothesis that endothelial cells constitute an additional FVIII source. Several subtypes of endothelial cells have been recently identified that secrete substantial amounts of FVIII.[16-19] Interestingly, part of FVIII synthesized by endothelial cells is co-stored with VWF in WPBs.[19] In the previous chapter we have shown that co-storage of FVIII in WPBs alters the organization of VWF tubules, which leads to impaired recruitment of platelets. These findings indicate that FVIII associates with VWF inside WPBs. The objective of the present study was to determine whether FVIII is able to interact with VWF strings released from WPBs, and if so, whether these strings can recruit FVIII from the FVIII-VWF complex.

Materials and Methods

Materials

All chemicals used were of analytical grade. The μ-slide I 0.4 Luer ibiTreat flowchambers were from Ibidi (Munchen, Germany). Human serum albumin (CeAlb) was from Sanquin (Amsterdam, the Netherlands). RPMI1640, M199, DAPI and anti-GFP were obtained from Invitrogen (Breda, the Netherlands). DMEM-F12, EGM-2 bullet kit, penicillin and streptomycin were from Lonza (Walkersville, MD, USA). Collagen type 1 rat tail was from BD Biosciences (Uppsala, Sweden). Peroxidase-labeled polyclonal rabbit anti-human VWF antibody was from DAKO (Glostrup, Denmark). Tissue culture flasks, multidishes and microtiterplates (Maxisorp) were from Nunc (Roskilde, Denmark).

Purification of recombinant proteins

Recombinant VWF production and purification has been described elsewhere.[20] VWF preparation characteristics were as described.[21] Stable cell lines of HEK293 expressing FVIII-YFP or FVIII-YFP Y1680F were produced as described before.[22] A monoclonal IgG_1 antibody of human antibody VK34[23] (directed against the FVIII heavy chain) was generated as described previously.[24] Recombinant FVIII and FVIII Y1680F were purified from conditioned medium by immunoaffinity chromatography employing human monoclonal antibody VK34 coupled to CNBr-Sepharose 4B. FVIII was eluted using 50 mM imidazole (pH 6.4), 40 mM $CaCl_2$, 55% (v/v) ethyleneglycol. Fractions containing FVIII were diluted 1:1 in 100 mM NaCl, 50 mM Tris (pH 8.8), 5 mM $CaCl_2$, 10% (v/v) glycerol and absorbed to Q Sepharose FF (Amersham Biosciences). FVIII was eluted by addition of 0.8 M NaCl, 50 mM Tris (pH 7.4), 10% (v/v) glycerol, 5 mM $CaCl_2$. Fractions were analyzed on YFP emission using a fluorescent microplate reader (Synergy HT, Bio-Tek Instruments, Winooski, VT, USA) and on total protein content by the method of Bradford.[25] FVIII-YFP containing fractions were dialyzed against 50 mM Tris (pH 7.4), 0.8 mM NaCl, 5 mM $CaCl_2$, 50% (v/v) glycerol and stored at -20°C. Protein concentration was determined by the method of Bradford. The FVIII concentration was determined with an enzyme-linked immunosorbent assay essentially as described.[26] FVIII activity was determined with a chromogenic assay according to the manufacturer's instructions (Chromogenix). Purified FVIII preparations were analyzed by 7.5% SDS-PAGE under reduced conditions followed by silver staining.

Live cell imaging of flow experiments

Production of viral vectors and BOEC isolation have been described elsewhere.[27] Lentiviral transduction of BOECs with FVIII-GFP, FVIII-YFP and FVIII-YFP Y1680F has been previously described (Chapter 3). BOECs were grown in collagen-coated flow chambers. EGM-20 medium (EGM-2 medium supplemented with EGM bullet kit and 20% FCS) was refreshed every other day until cells were confluent. SF medium supplemented with 100 μM histamine was perfused over the cells at 2.5 dynes/cm^2 for 10 minutes. SF medium containing 200×10^9 washed platelets/l, SF medium supplemented with 5 U/ml purified FVIII-YFP or 5 U/ml FVIII-YFP Y1680F was perfused over the cells at the same shear rate. Live-cell imaging was performed at 37°C on a Zeiss LSM510 equipped with Plan NeoFluar 40x/1.3 Oil objective (Carl Zeiss, Heidelberg, Germany). Images were taken using Zen 2009 LE software (Carl Zeiss, Heidelberg, Germany) at 10-second intervals for indicated time ranges after the onset of perfusion with histamine, unless indicated otherwise. Images were processed and analyzed with ImageJ Software (Rasband, U.S. National Institutes of Health, Bethesda, MD, USA, http://rsb.info.nih.gov/ij/, 1997-2009).

Immunofluorescent analysis of flow experiments

Confluent BOECs grown in collagen-coated flow chambers were stimulated with SF medium supplemented with 100 μM histamine by perfusion at 2.5 dynes/cm^2 for 2 minutes followed by static incubation for 6 minutes, after which flow was continued for an additional 2 minutes. When indicated, cells were perfused with SF medium supplemented with purified FVIII or FVIII Y1680F at 5 U/ml for 5 minutes. Alternatively, for binding of FVIII in presence of soluble VWF, purified FVIII was incubated for 10 minutes in 0.5 ml SF medium with recombinant VWF at indicated molar ratios, diluted in SF medium to a final concentration of 1.5 nM FVIII and perfused over the cells for 5 minutes. Cells were washed with PBS at 2.5 dynes/cm^2 for 1 minute and fixed with 3.7% PFA by incubating 2 minutes under flow, 6 minutes under static conditions and 2 minutes under flow. Cells were washed with PBS and incubated for 1 hour with rabbit anti-human VWF antibody (DAKO, Glostrup, Denmark) and FITC-labelled CLB-CAg9[28] (directed against FVIII heavy chain) in PBS, 1% BSA. Alexa-633 conjugated antibody was used as secondary antibody to detect VWF. Cells were mounted in Mowiol 4-88 and stored at 4°C until use. Cells were viewed on a Zeiss LSM510 equipped with Plan NeoFluar 40x/1.3 Oil objective (Carl Zeiss, Heidelberg, Germany). All experiments were performed at 37°C.

Results

FVIII released by endothelial cells binds to VWF strings

We found that FVIII expression alters several properties of VWF stored in WPBs, including disruption of VWF tubules in WPBs, decrease of VWF multimer size, and reduction of platelet adhesion to VWF strings once released from WPBs (Chapter 4). This strongly suggests that FVIII assembles with VWF inside endothelial cells. To determine whether FVIII and VWF are also associated after release from WPBs, we monitored the release of FVIII from WPBs of FVIII-expressing BOECs in real-time. Following stimulation with histamine, FVIII-containing WPBs fused with the plasma membrane and patches of released FVIII were observed (Figure 1, time point 1). Surprisingly, these patches did not diffuse but extended to form fluorescent strings (Figure 1; see arrowheads). This indicates that FVIII released from WPBs binds to the secreted VWF strings. In fact, released UL-VWF strings remained covered with FVIII throughout the entire experiment (15 min).

Next we addressed the question as to whether FVIII needs to be pre-assembled with VWF inside WPBs in order to bind to VWF strings. Non-transduced BOECs were stimulated with histamine followed by perfusion with purified FVIII to determine whether exogenous FVIII is able to bind to VWF strings under flow. Indeed, purified FVIII was capable of binding to UL-VWF strings without pre-storage with VWF in WPBs (Figure 2A). In addition, exogenous FVIII was also able to bind to VWF strings that were already covered with platelets (Figure 2B). Binding of platelets to FVIII-positive filament also confirmed that these were truly UL-VWF strings. Surprisingly, while we demonstrated previously that endogenously secreted FVIII interferes

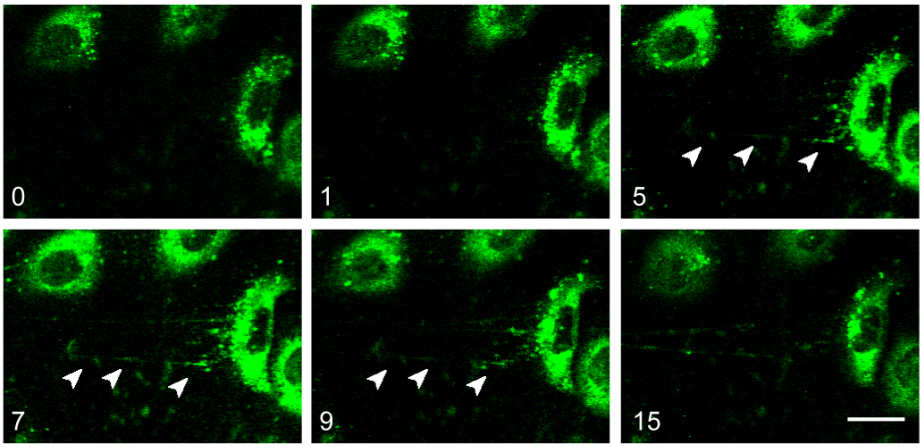

Figure 1. FVIII-containing, rounded WPBs release UL-VWF strings covered with FVIII. (A) FVIII-transduced BOECs were stimulated with 100 µM histamine at 2.5 dynes/cm2. Shown are fluorescent images taken at indicated time points (in minutes) after the onset of flow. FVIII fluorescence is depicted in green; arrowheads mark released patches and strings. Live-cell imaging was performed at 37°C on a confocal laser scanning microscopy using a Zeiss LSM510 equipped with Plan NeoFluar 40x/1.3 Oil objective (Carl Zeiss, Heidelberg, Germany) at 37°C. Scale bar represents 20 µm.

with platelet binding (Chapter 3), VWF strings covered with exogenous FVIII did display platelet binding (Figure 2C). Quantification of the number of strings that bound to VWF strings covered with exogenously added FVIII demonstrated that the presence of exogenous FVIII did not interfere with platelet binding to any appreciable extent (Figure 2D). This shows that newly released UL-VWF strings have the capacity to recruit both platelets and exogenous FVIII.

FVIII Y1680F remains associated with released VWF strings

The observation that WPB-derived FVIII (Chapter 4), but not exogenous FVIII, effectively reduces platelet binding suggests a difference in FVIII binding to VWF strings, for instance in

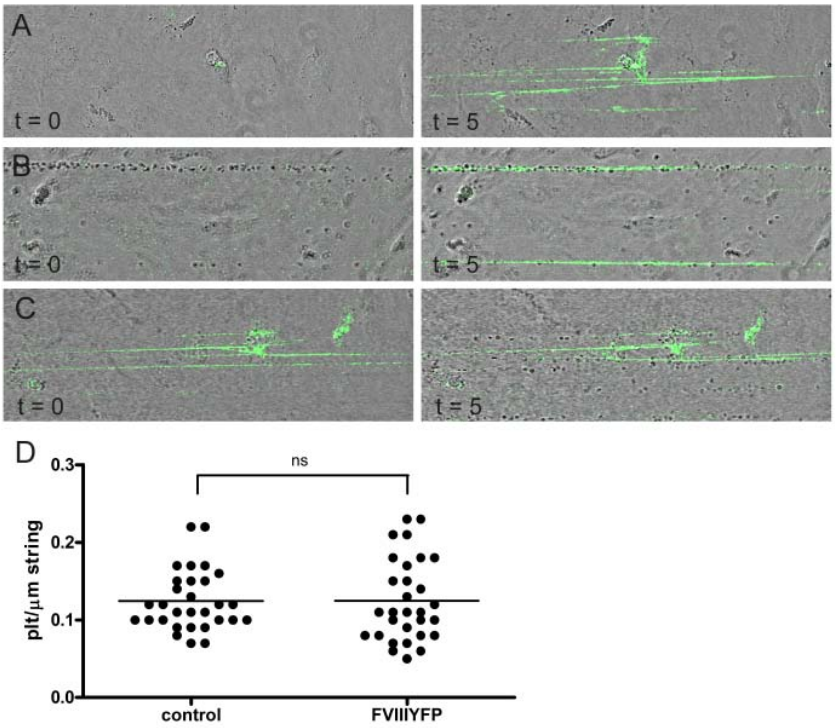

Figure 2. Exogenously added FVIII binds to VWF strings released by non-transduced BOEC. (A) Non-transduced BOECs were stimulated with 100 μM histamine at 2.5 dynes/cm^2 for 10 minutes to stimulate exocytosis of WPBs and subsequently incubated with 5 U/ml purified FVIII-YFP for 5 minutes. Images were taken at the indicated time points (in minutes) after the onset of FVIII-YFP perfusion. (B) Non-transduced BOECs were stimulated with 100 μM histamine at 2.5 dynes/cm^2 for 10 minutes and subsequently incubated with washed platelets for 5 minutes before addition of 5 U/ml purified FVIII-YFP for 5 minutes. (C) Non-transduced BOECs were stimulated with 100 μM histamine at 2.5 dynes/cm^2 for 10 minutes, incubated with 5 U/ml purified FVIII-YFP for 5 minutes followed by perfusion of washed platelets for 5 minutes. Representive pictures of at least 3 experiments are shown. Live-cell imaging was performed at 37°C on a confocal laser scanning microscopy using a Zeiss LSM510 equipped with Plan NeoFluar 40x/1.3 Oil objective (Carl Zeiss, Heidelberg, Germany) at 37°C. (D) Non-transduced BOECs were stimulated with 100 μM histamine at 2.5 dynes/cm^2 for 10 minutes, incubated with SF medium (control) or 5 U/ml purified FVIII-YFP (rFVIII) for 5 minutes followed by perfusion of washed platelets for 5 minutes. Number of platelets per string was determined of 30 strings each. No difference was observed in platelet binding between strings covered with or without recombinant FVIII ($p=0.98$).

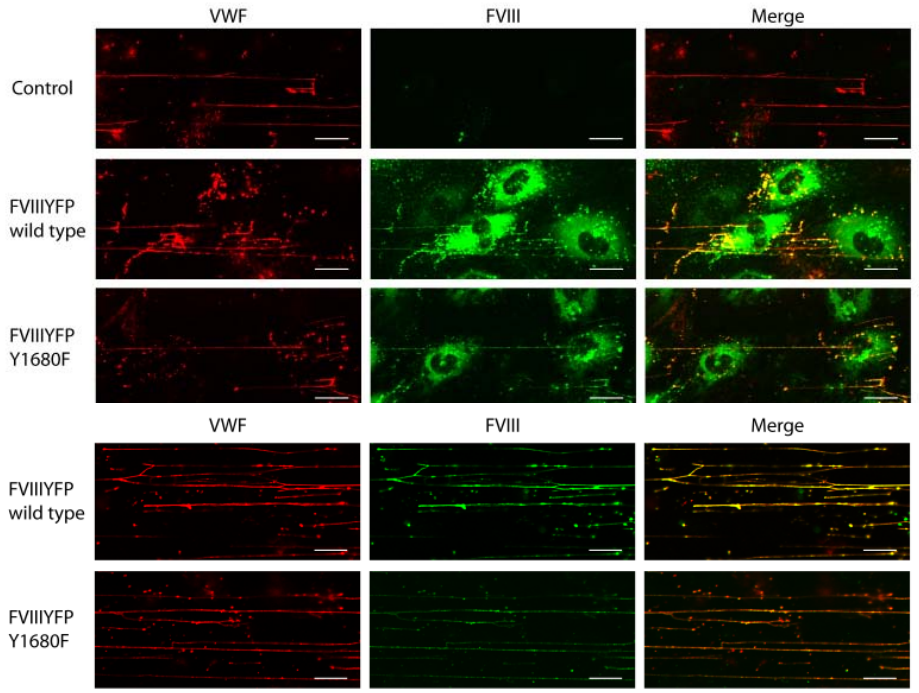

Figure 3. FVIII Y1680F variant binding to VWF strings. (A) Non-transduced, FVIII-, and FVIII Y1680F-transduced BOECs were stimulated with 100 μM histamine at 2.5 dynes/cm^2, fixed and stained for VWF (red) and FVIII (green). Representive pictures of at least 3 experiments are shown. Scale bars represent 20 μm. (B) Non-transduced BOEC were stimulated with 100 μM histamine at 2.5 dynes/cm^2 followed by perfusion with 5 U/ml purified FVIII or 5 U/ml FVIII Y1680F. Cells were fixed and stained for VWF (red) and FVIII (green). Representive pictures of at least 3 experiments are shown. Scale bars represent 20 μm.

terms of affinity. We addressed this issue by assessing the effect of the FVIII Y1680F variant, which lacks the high-affinity interaction with VWF.[13,20] Histamine-treated endothelial cells were fixed and stained for VWF to also allow detection of VWF strings that had not bound FVIII. Because of its strongly reduced affinity for VWF, we anticipated that FVIII Y1680F released from BOECs would not bind to VWF strings or dissociate quickly after exocytosis. FVIII Y1680F released by endothelial cells remained detectable on VWF strings, however, and the fluorescence intensity was similar to that of strings covered with normal FVIII (Figure 3). To determine whether binding of FVIII Y1680F to strings is dependent on FVIII-VWF co-storage in WPBs, purified FVIII Y1680F was perfused over histamine treated BOECs. Exogenously added FVIII Y1680F did bind to VWF strings but the intensity of the FVIII fluorescent signal was reduced compared to exogenously added normal FVIII (Figure 3). On the other hand, the fluorescence signal for VWF was identical in both samples, which indicates that the reduced binding of FVIII Y1680F was not the result of differences in the released VWF strings. This qualitative evidence suggests that, in contrast to 'endogenous' FVIII from WPBs, binding of exogenous FVIII to VWF strings may be more dependent on high-affinity, Y1680-mediated interaction with VWF.

VWF strings bind FVIII in the presence of soluble VWF

While purified FVIII perfused over stimulated endothelial cells is able to bind newly released VWF strings, it is not clear whether FVIII can bind to VWF strings in the presence of soluble VWF. Therefore, we pre-incubated FVIII with a concentration range of recombinant VWF and perfused these mixtures over histamine-stimulated endothelial cells (Figure 4). For estimating the fractions of free FVIII in these mixtures, we assumed that normal FVIII and VWF monomer plasma concentrations of 1 U/ml correspond with 0.3 nM and 50 nM, respectively,[29,30] and a K_d between 0.2-0.4 nM.[12,31] As anticipated, FVIII without soluble VWF present bound readily to the released VWF strings. All VWF strings were homogenously covered with FVIII, as indicated by the yellow color in the merge picture. While at a 1:1 FVIII-VWF molar ratio an estimated 20-30% of FVIII is not bound to soluble VWF, FVIII bound to VWF strings similar to control in our qualitative set-up. However, a shift from yellow to orange fluorescent signal in the merge picture indicated that FVIII binding to strings decreased significantly at higher VWF ratios. At the highest FVIII to VWF ratio tested (1:32; estimated to contain 0.6-1.2% unbound FVIII), we could still detect FVIII fluorescence on VWF strings. These data demonstrate that UL-VWF strings are able to bind appreciable quantities of FVIII in the presence of soluble VWF.

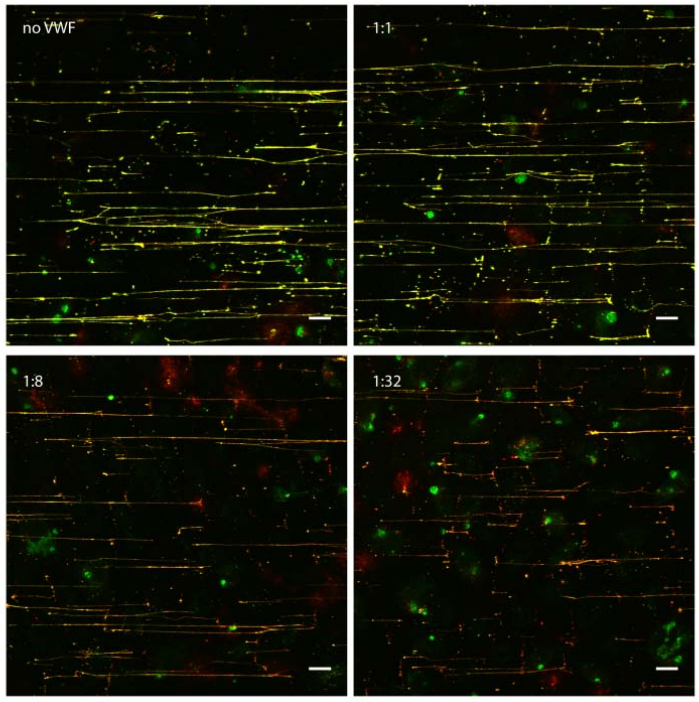

Figure 4. Binding of FVIII to strings in presence of soluble VWF. Purified FVIII was incubated for 10 minutes recombinant VWF at indicated molar ratios. Prior to perfusion, FVIII-VWF mixtures were diluted in SF medium to a final concentration of 1.5 nM FVIII. Non-transduced BOEC were stimulated with 100 μM histamine at 2.5 dynes/cm^2 followed by perfusion of mixtures of 1.5 nM FVIII with recombinant VWF at indicated molar ratios for 5 minutes. Cells were fixed and stained for VWF (red) and FVIII (green). Representive pictures of at least 3 experiments are shown. Scale bars represent 20 μm.

Discussion

FVIII expressed in endothelial cells is stored with VWF in WPBs.[32] WPB exocytosis can be induced by several stimuli such as histamine, epinephrine or phorbol 12-myristate 13-acetate (PMA). Exocytosis of FVIII-containing WPBs may release of FVIII in complex with VWF. Recently we demonstrated that high-affinity FVIII-VWF binding sites are dispensable for sorting of FVIII to WPBs.[20,21] Mutations in either FVIII or VWF leading to impaired complex formation did not alter sorting of FVIII. In this study we show that FVIII secreted from WPBs does not diffuse freely in solution but binds to released VWF strings (Figure 1). While exogenously added normal FVIII also binds to VWF strings released from non-transduced BOECs (Figure 2), exogenous FVIII Y1680F is less able to bind VWF strings than FVIII Y1680F released from WPBs (Figure 3). These findings suggest that the interaction of FVIII with VWF within and during formation of WPBs differs from that in the circulating FVIII-VWF complex. In addition, the similarities between endogenously released normal FVIII and FVIII Y1680F also indicate that intracellular FVIII-VWF assembly involves other molecular sites in FVIII, and possibly also in VWF, besides those previously established for these proteins in circulation. This might explain why VWF variants with reduced FVIII binding in the circulation are still able to target FVIII to WPBs upon co-expression in endothelial cells.[21] We therefore propose that structural elements that play a minor role in extracellular FVIII-VWF complex assembly do contribute considerably to intracellular assembly of FVIII to VWF.

Although binding sites of FVIII for circulating VWF have been well characterized, we question whether FVIII binds to VWF strings in a similar manner. Multimers in VWF strings are not only much larger due to extensive multimerization inside WPBs but also have a different conformation from circulating VWF. The conformation of VWF changes considerably when shear stress is applied to the protein. Circulating VWF subjected to low shear has a globular conformation, in which the D'-D3 region is presumably associated with the A1 domain to prevent platelets from spontaneously adhering to VWF.[33,34] At high shear rates, the molecule stretches and platelets can bind VWF, which suggests that shear exposes the A1 domain with the GP1bα binding site.[35] Besides the suggested exposure of VWF A1 domain, shear stress also reveals the metalloprotease ADAMTS-13 cleavage site in the A2 domain.[36] As binding sites for the VWF-cleaving ADAMTS13 and GP1bα on platelets are only exposed when VWF is unfolded, it would be possible that binding sites for FVIII also differ in globular VWF from those in strings. Shear stress might break interactions between the D'-D3 region and A1 domain, thereby further exposing the FVIII binding site. The shear stress in our experiments was only 2.5 dynes/cm^2, and thus too low to change to conformation of soluble VWF.[33] As the VWF used for the flow experiments depicted in Figure 4 can be assumed to be in a globular conformation, we qualitatively compared FVIII binding to stretched and globular VWF. Although FVIII perfused over VWF strings is able to bind these strings in the presence of a 32-fold excess of soluble VWF (Figure 4), we did not detect more FVIII on VWF strings than may be expected when considering the K_d-value of the FVIII-VWF complex. As the K_d ranges from 0.2 to 0.4 nM[12,31], approximately 1% of FVIII remains available for binding to VWF strings at a 1:32 molar ratio.

Although we anticipated differences in affinity for FVIII of strings and globular VWF, it seems that our qualitative system is not suitable to detect (subtle) affinity changes. This is illustrated in Figure 4 panel '1:1' ratio. While here the fraction of unbound FVIII should be reduced by more than 50%, we observed a similar fluorescence FVIII signal on the strings (compare Figure 4; panel 'no VWF' and '1:1 ratio'). Therefore, it would be interesting to use a quantitative approach to determine potential differences in binding affinity of FVIII for globular or stretched VWF. If VWF strings have an increased affinity for FVIII, VWF strings secreted at sites of vascular damage could serve as a dissociation signal for the FVIII-VWF complex to give a local rise in FVIII levels. Based on the assumption that the plasma concentration is 0.3 nM for FVIII[30] and 50 nM for VWF monomer[29], the molar ratio of FVIII:VWF in circulation is approximately 5-fold more than the highest concentration used in this study (Figure 4). Therefore it remains unclear whether FVIII can bind to VWF strings released at sites of vascular injury. Interestingly, using a mathematical model of FVIII kinetics Noe estimated that plasma contains approximately 5% unbound FVIII when the FVIII and VWF levels are equal to 1 U/ml.[37] Therefore an alternative model would be that VWF strings are able to recruit the unbound FVIII fraction from circulation, thereby increasing local FVIII levels at sites of vascular damage. FVIII associated on VWF strings could then be activated by traces of thrombin to support localized propagation of the coagulation cascade.

Acknowledgements

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Chapter 6

Role of factor VIII C domains in sorting to Weibel-Palade bodies

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Abstract

Recent studies have shown that factor VIII (FVIII) expressed in endothelial cells sorts with von Willebrand factor (VWF) to secretory Weibel-Palade bodies (WPBs). The sorting mechanism remains controversial although VWF is thought to be essential. However, mutations that lead to impaired FVIII-VWF complex assembly do not reduce the sorting efficiency of FVIII. As factor V (FV) and FVIII are highly homologous in structure, we addressed the possibility that FV sorts to WPBs as well. Blood outgrowth endothelial cells (BOECs) were transduced with lentiviral vectors encoding FV, FVIII deletion mutants, or FVIII-FV chimeras. We found by confocal microscopy and subcellular fractionations that FV displays a strong reduction in sorting efficiency compared to FVIII. This indicates that sorting to WPBs is mediated by FVIII-specific structural elements. As the C domains of FVIII are implicated in membrane and VWF binding, these domains could drive sorting to WPBs. Therefore we constructed FVIII variants lacking C domains to establish their role in WPB sorting. Quantitative determination of the sorting efficiencies demonstrates that the C1 domain is not of major importance for sorting to WPBs, whereas the C2 domain is. Moreover, exchanging the FVIII C domains for corresponding domains of FV also suggests that the C2 domain drives WPB sorting. This leads to the conclusion that FVIII sorting to WPBs is driven by FVIII-specific structural elements in both C domains, but in particular the C2 domain.

Introduction

Factor VIII (FVIII) serves as a co-factor for activated factor IX in the FX-activating complex. It consists of 2332 amino acids with a distinct domain structure A1-a1-A2-a2-B-a3-A3-C1-C2.[1] Intracellular cleavage of the B domain yields a heterodimeric FVIII protein with a 90-220 kDa heavy chain (A1-a1-A2-a2) non-covalently attached to a 80 kDa light chain (a3-A3-C1-C2).[2] FVIII circulates in complex with the multimeric glycoprotein von Willebrand factor (VWF) that protects FVIII from premature clearance and proteolytic degradation.[3] Complex assembly occurs over an extended surface on FVIII, spanning the entire light chain.[4-7] The sulfated tyrosine on position 1680 is essential for binding to VWF and mutation of this tyrosine results in impaired complex formation with VWF.[4]

Recently is has been shown that FVIII expressed in endothelial cells co-sorts with VWF to the secretory organelles Weibel-Palade bodies (WPBs).[8-10] The precise interaction mediating sorting to WPBs has not been clarified, although it has been generally assumed that VWF plays a key role as sorting chaperone. In contrast to this view, we have shown that FVIII sorting to WPBs does not require a high-affinity interaction of FVIII with VWF via the sulfated tyrosine on position 1680.[11] Also mutations in VWF leading to impaired extracellular complex assembly do not influence sorting of FVIII to WPBs.[12] Therefore, the role of VWF in FVIII sorting to WPBs has remained controversial.

Factor V (FV) shares ~40% sequence homology with FVIII and has a similar domain structure (A1-A2-a2-B-a3-A3-C1-C2).[13,14] FV functions as a co-factor for FXa in the prothrombinase complex, demonstrating that FVIII and FV serve a similar co-factor function. Considering the structural and functional similarities between FVIII and FV, we addressed the possibility that also FV could sort to WPBs. Our study was designed to identify domains in FVIII that are needed for sorting to WPBs by means of domain deletions and FVIII-FV domain exchange. As the C domains of FVIII contain membrane and VWF binding sites, we particularly focused on comparing the C domains of FVIII and FV.

Materials and methods

Materials

All chemicals used were of analytical grade. Fetal calf serum was from Hyclone (Logan, UT, USA). One shot *Stbl3* chemically competent cells, trypsin, and DMEM (4.5 g/L glucose) were obtained from Invitrogen (Breda, the Netherlands). DNA modifying enzymes were from NEB (Ipswich, MA, USA). EGM-2 bullet kit, penicillin and streptomycin were from Lonza (Walkersville, MD, USA). Collagen type 1 rat tail was from BD Biosciences (Uppsala, Sweden). Peroxidase-labeled polyclonal rabbit anti-human VWF antibody was supplied by DAKO (Glostrup, Denmark). Tissue culture flasks, multidishes and microtiterplates (Maxisorp) were from Nunc (Roskilde, Denmark).

Construction of FVIII variants

FVIIIdB variants lacking the C1, C2 or C1C2 domains in pcDNA3.1(+) (Invitrogen) were created by removal of the domains from pcDNA3.1(+) FVIIIdB[12] by Quick Change (Stratagene) mutagenesis using appropriate primers. For construction of FVIII with FV C1 or C2 domain the FV C1 domain was amplified from pcDNA3.1(+) encoding B domain deleted FV 811-1491[15] using primers (sense) 5′-ATAACCGGTGGACTAAGCACTGGTATC-3′ and (antisense) 5′-TATTGGCGCGCCACCATTTACCTCACAACCTTG-3′, FV C2 domain with primers (sense) 5′-ATAACGGTGGATGTT CCACACCCCTG-3′ and (antisense) 5′-TATTGGCGCGCCACCGTAAATATCACAGCCAAA-3′. PCR products were ligated in pGEM-T Easy vector (Promega) and digested with *AgeI* and *AscI*. The FVIII C1 or C2 domain in pcDNA3.1(+) FVIIIYFP[11] were replaced for *AgeI-AscI* restriction sites by Quick Change mutagenesis using primers C1 (sense) 5′-CTGGTGTACAGCAATAAGACCGGTGGCGCGCCAAGTTGCAGCATGCCATTG-3′, C1 (antisense) 5′-CAATGGCATGCT GCAACTTGGCGCACCGGTCTTATTGCTGTACACCAG-3′, C2 (sense) 5′-ATGGGCTGTGATTTAAATACCGGTGGCGCG CCATGAGGGTGGCCACTGCAG-3′ and C2 (antisense) 5′-CTGCAGTGGCCACCCTCATGGCGCGCCACCGGTATTTAAA TCACAGCCCAT-3′. Constructs were digested with *AgeI-AscI* and ligated with corresponding FV C domain fragments. *AgeI* and *AscI* restriction sites bordering the C domains were removed by Quick Change mutagenesis. FVIIIdB with 2092/2093/2199/2200/2251/2252A was constructed from pcDNA3.1(+) FVIIIdB 2092/2093A[16] (kindly provided by dr. H. Meems) by Quick Change mutagenesis using appropriate primers.

Construction of lentiviral vectors

The pLV-CAG-FVIIIdB plasmid encoding B-domain deleted FVIII (FVIIIdB) and mutants thereof were created by replacing fragment *NheI-XhoI* from pLV-CAG-FVIIIGFP vector[9] for the corresponding fragment of the pcDNA3.1(+) plasmids. To clone B-domain deleted FV (FVdB) in lentiviral vector, pLV-CAG-FVIIIGFP vector was first digested with *NheI* followed by partial digestion with *NotI*. Briefly, *NheI*-digested construct was mixed on ice with serial dilutions of *NheI*, incubated for 15 minutes at 37°C and heated to 65°C for 20 minutes to inactivate the restriction enzyme. The fragment corresponding to lentiviral vector without insert was isolated from gel and ligated with the *NheI-NotI* fragment of FVdB in pcDNA3.1(+). Production of viral vectors, BOEC isolation and transduction with the viral vectors have been described before.[9]

Immunofluorescence microscopy

BOECs were grown on collagen-coated 1 cm-diameter glass coverslips. Confluent cells were fixed with 3.7% PFA in PBS for 15 minutes at room temperature. After fixation, the cells were labeled with monoclonal antibody CLB-RAg20[17] to detect VWF, followed by incubation with Alexa-633 conjugated secondary antibody and FITC-labeled

EL-14[12] (directed against FVIII C2 domain), FITC-labeled KM33[16] (against FVIII C1 domain) or FITC-labeled CLB-FV 4 (directed against FV light chain). Cells were embedded in 4-88 Mowiol and stored at 4°C until analysis. Z-stacks were taken with a Zeiss LSM510 equipped with Plan NeoFluar 63x/1.4 Oil objective (Carl Zeiss, Heidelberg, Germany).

Subcellular fractionation

Fractionation of transduced BOECs and quantification of FVIII and VWF antigen levels have been described elsewhere.[9] For determination of FV antigen, fractions were serially diluted in TBS, 0.1% (v/v) Tween-20, 1% (v/v) HSA and incubated for 2 hours at 37°C on a 96-wells plate coated with monoclonal CLB-FV 4. Plates were washed with PBS, 0.1% (v/v) Tween-20 and incubated with peroxidase-labeled polyclonal anti-FV (Affinity Biologicals, Ancaster, Canada) diluted in TBS, 0.1% (v/v) Tween-20, 0.1% (v/v) HSA for 1 hour at 37°C. Purified FVdB with known concentration was used as standard. Storage efficiency was calculated as the percentage of protein in the dense WPB fractions of the total intracellular protein content. Total intracellular and WPB molar ratios were calculated by dividing the amount of FVIII/FV by the amount of VWF in the corresponding fractions.

Modeling of FVIII variants

All FVIII models were based on the crystal structure of FVIII (PDB code 2r7e).[18] To generate models of the FVIIIdelC1 and FVIIIdelC2, residues 2021 to 2172 or residues 2173 to 2332 were deleted from the file, respectively (protein domains according to Swiss-Prot entry P00451). For the FVIII FV C1 and FV C2 models the same residues were replaced by residues 1877 to 2034 and residues 2035 to 2194 of the FV sequence, respectively (protein domains according to Swiss-Prot entry P12259). Models were generated with the Modeller 9v7 freeware package and analyzed with MolMol 2K.2 software.

Results

Factor V sorting to WPBs is reduced compared to factor VIII

Because FV is structurally highly homologous to FVIII, we compared FVIII and FV with respect to sorting to WPBs in BOECs. FVIII expressed in endothelial cells co-localized with VWF in WPBs (Figure 1A). FVIII-containing WPBs were round, while WPBs negative for FVIII retained the typical cigar-shaped appearance. Although FV is normally synthesized in megakaryocytes[19], FV-transduced BOECs were able to produce high amounts of FV (approx. 140 pmol/1×10^6 cells/72 h). In contrast to FVIII expressed in endothelial cells, confocal microscopy revealed that FV did not sort to WPBs although intracellular staining for FV could be detected (Figure 1A). While most WPBs did not contain detectable amounts of FV, a minority stained positive for FV though the shape of these WPBs remained elongated (see inset FV, Figure 1A). Subcellular fractionation confirmed at a quantitative level that some FV is indeed stored in WPBs (Figure 1B). Although the total intracellular molar ratio of FV to VWF was 3:1, the ratio in WPBs was only approximately 1:6 (Table 1). This was in contrast with FVIII that had more or less the same FVIII:VWF ratio both intracellular (1:60) and inside WPBs (1:45). Also when the sorting efficiency for both proteins was calculated, the efficiency for FV was significantly reduced: approximately 20% of total intracellular FVIII sorted to the WPBs versus 2% for FV, though the amount of VWF stored in WPBs was comparable (Table 1). The low sorting efficiency of FV implies that sorting to WPBs is specific for structural elements in FVIII.

Deletion of FVIII C domains decreases sorting to WPBs

The observed discrepancy in sorting of FVIII and FV may indicate that an interaction with VWF is essential for sorting to WPBs. As the C domains of FVIII are known to contribute to VWF binding, we expressed a FVIII variant lacking both C domains (FVIIIdelC1C2) in endothelial cells. Even though both C domains were removed, small quantities of the FVIII mutant could be detected in conditioned medium (~0.03 pmol/1×10^6 cells/72 h). This demonstrates that FVIIIdelC1C2 is not only synthesized by endothelial cells but also secreted. While the FVIIIdelC1C2 mutant is secreted by endothelial cells, we did not observe any FVIII localizing in WPBs (Figure 2A). This observation indicates that one or both of the C domains are needed to sort FVIII to WPBs. To determine which domain is involved in sorting, we made two FVIII variants; one lacking the C1 domain (FVIIIdelC1) and one lacking the C2 domain (FVIIIdelC2). Surprisingly, despite the deletion of an entire domain, the FVIIIdelC1 mutant was efficiently

	Percentage VWF in WPBs	Sorting efficiency FVIII or FV	Intracellular ratio to VWF	WPB ratio to VWF
FVIII	18%	23%	1:61	1:47
FV	35%	2.1%	3:1	1:6

Table 1. Sorting efficiency to WPBs of FVIII and FV. Subcellular fractionations were performed on BOECs expressing either FVIII or FV. Representative graphs of the fractionations are displayed in Figure 1B. The intracellular and WPB ratio, and the percentage of protein inside WPB fractions were calculated as described in the Methods section. Fractionations were performed once for FVIII and twice for FV.

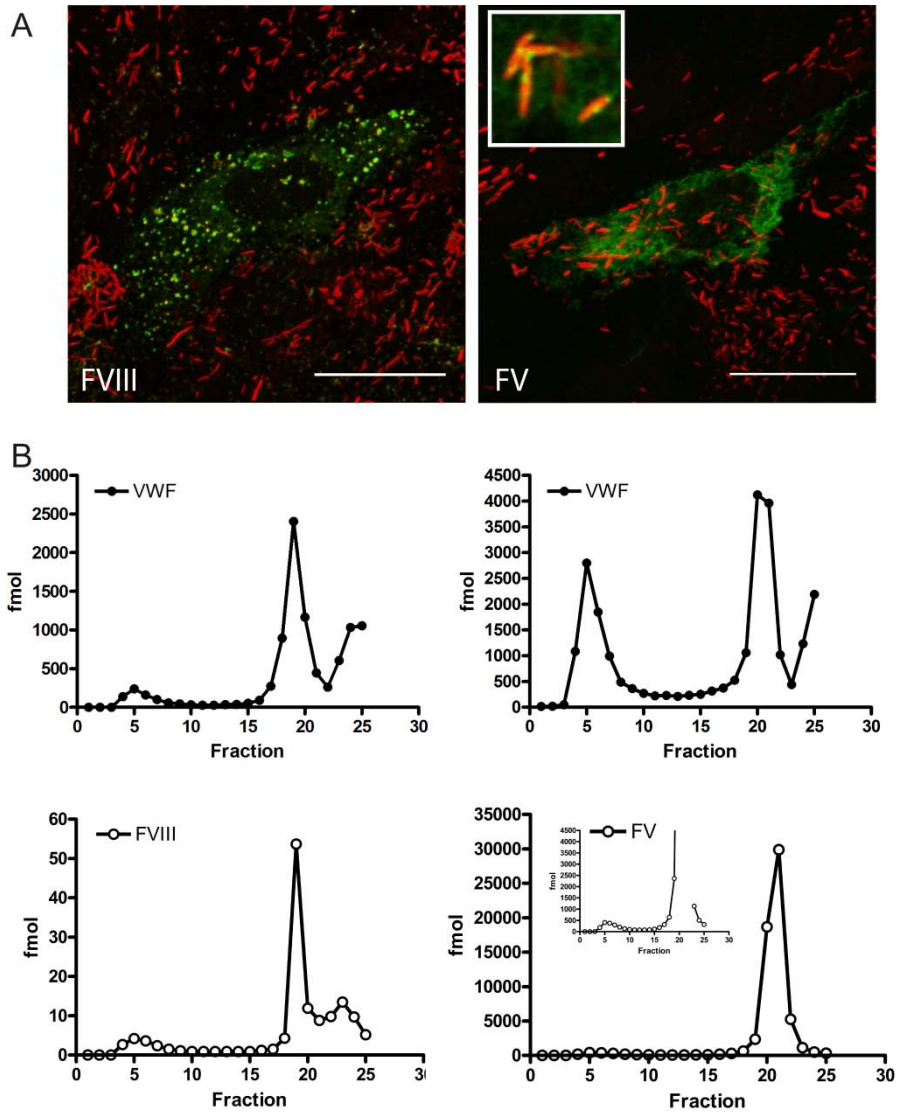

Figure 1. Sorting of FVIII and FV to WPBs. (A) Confocal images of BOECs expressing FVIII (left; in green) or FV (right; in green) stained for VWF, shown in red. Yellow indicates co-localization of VWF with FVIII or FV in WPBs. FVIII is stored with VWF in WPBs, whereas hardly any FV is sorted to the WPBs. While FVIII-containing WPBs have a round morphology, FVIII-negative WPBs remain elongated. FV storage does not change the appearance of WPBs (inset). (B) FVIII and FV storage in WPBs was quantified by subcellular fractionations. VWF antigen (upper panels) and FVIII (left bottom panel) or FV (right bottom panel) antigen in the various fractions were quantified by ELISA. The first peak corresponds to the dense WPB fraction, whereas the second peak contains ER, Golgi apparatus and constitutively released vesicles.

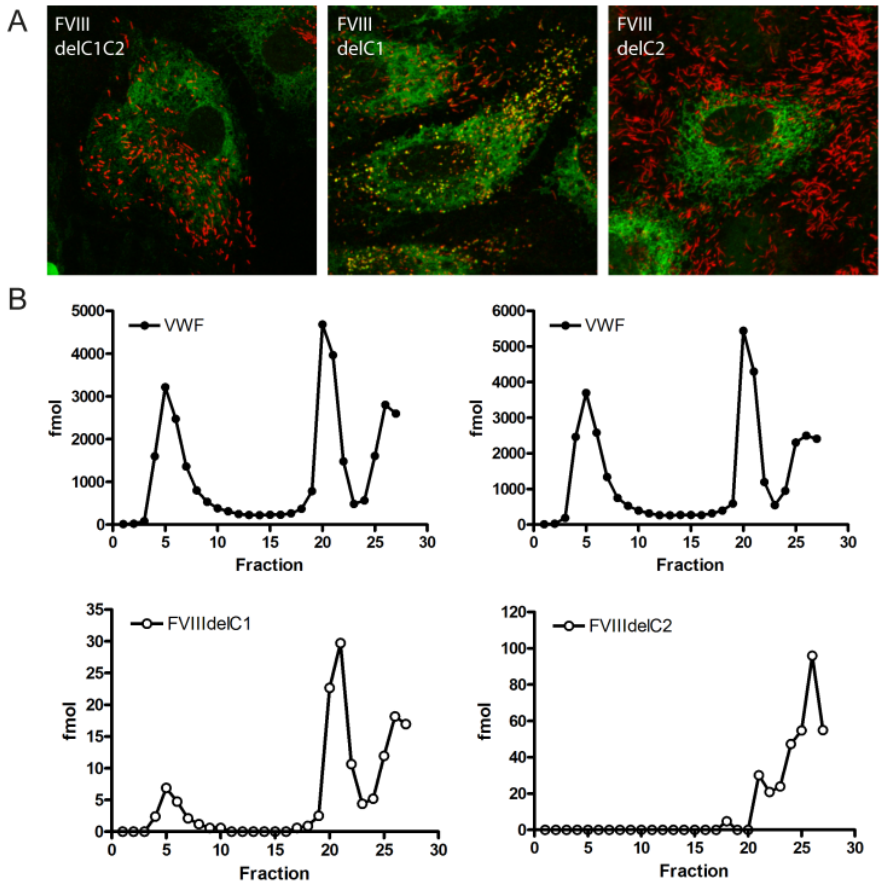

Figure 2. Sorting of FVIII deletion mutants to WPBs. (A) FVIII without both C domains (FVIIIdelC1C2), FVIII without the C1 domain (FVIIIdelC1), and FVIII without the C2 domain (FVIIIdelC2) were expressed in BOECs. Cells were stained for FVIII (green) and VWF (red). Co-localization of FVIII with VWF is indicated by yellow merge of both signals. (B) Subcellular fractionations were performed to quantify the sorting efficiency of FVIIIdelC1 (left panel) and FVIIIdelC2 (right panel). VWF antigen (upper panels) and FVIII antigen (lower panels) were determined by ELISA. FVIIIdelC1 was detected using primary antibody CLB-CAg9[17] and HRP-labeled CLB-CAg117[32]. FVIIIdelC2 was detected with CLB-CAg12[33] and HRP-labeled CLB-CAg9[17].

expressed as levels of approximately 3.2 pmol/1×10^6 cells/72 h were measured. In contrast, expression of the FVIII variant lacking the C2 domain was approximately 10-fold lower (~0.25 pmol/1×10^6 cells/72 h). These data indicate that both FVIII deletion mutants are expressed at sufficient levels for analysis of WPB sorting.

Using confocal microscopy we found that deletion of the C1 domain did not abolish FVIII sorting entirely, although a considerable number of FVIIIdelC1-expressing cells did not contain FVIII in WPBs (Figure 2A). The defect in WPB sorting was more pronounced when the

C2 domain was deleted as FVIII without the C2 domain did not sort to WPBs anymore. Quantification of the sorting efficiency of the FVIIIdelC1 and FVIIIdelC2 variants by subcellular fractionation confirmed these qualitative data (Figure 2B and Table 2). The sorting efficiency of the FVIIIdelC1 variant was 13%, which is slightly reduced compared to normal FVIII (23%). As expected, no FVIIIdelC2 could be detected in WPB fractions, in spite of the intracellular FVIII/VWF being 2-fold higher than for FVIIIdelC1 (Table 2). This indicates that the C1 domain of FVIII is expendable for sorting to WPBs, whereas the C2 domain is not.

As domain deletions can have a major impact on the protein conformation, we made models of FVIII lacking the C1 or C2 domain (Figure 3A-C). Figure 3B shows that the C2 domain could replace the C1 domain in the FVIIIdelC1 mutant, although none of the residues normally interacting with the A3 domain[18] are preserved. This implies that the C2 domain could be loosely tethered at the position where normally the C1 domain is located. As this indicates that the normal domain orientation is lost, the contribution of the C1 domain in WPB sorting remains inconclusive. Therefore, although we established that at least the C2 domain is involved in sorting FVIII to WPBs, an complementary approach was required to study the contribution of the separate C domains.

C domains of factor VIII contribute to co-trafficking with VWF

To determine which of the C domains contribute to sorting FVIII to WPBs, we exchanged the FVIII C domains for those of FV. Because of potential difficulties in staining of these variants with antibodies directed to the FVIII light chain, we used YFP-tagged FVIII in this experiment. Figure 4 shows models of FVIII with FV C1 or C2 domain substitutions. Herein, the FV domain orientation is almost identical to the original FVIII domains as was expected due to the structural homology of both proteins. Most residues interacting between the C1 and A3 or C2 and A1 are conserved, suggesting that the domain orientation within the molecule is not disturbed. In the FVIII FV C1 mutant four out of 16 residues interacting with either the A3 or C2 domain[18] are altered, which are T2023M, H2082T, T2122N, and I2139Q. For FVIII FV C2 two out of seven residues[18,20] are changed, namely M2176T and L2324F (Figure 4). However, expression levels of FVIII FV C1 and C2 variants varied considerably as levels of respectively 0.03±0.02 and

	Percentage VWF in WPBs	Sorting efficiency FVIII	Intracellular FVIII/VWF	FVIII/VWF in WPBs
FVIII del C1	36%	13%	1:200	1:550
FVIII del C2	37%	n.d.	1:100	-
FVIII FV C1	19%	n.d.*	1:3400*	-
FVIII FV C2	34%	2,7%	1:80	1:1000

Table 2. Sorting efficiency to WPBs of FVIII variants. Subcellular fractionations were performed on BOECs expressing FVIII variants (see also Figures 2B and 5B). The percentage of protein inside WPB fractions and the FVIII/VWF ratios were calculated as described in the Methods section. Fractionations were performed once for all mutants. *Signal may be out of detection range due to low expression level. N.d. = not detectable.

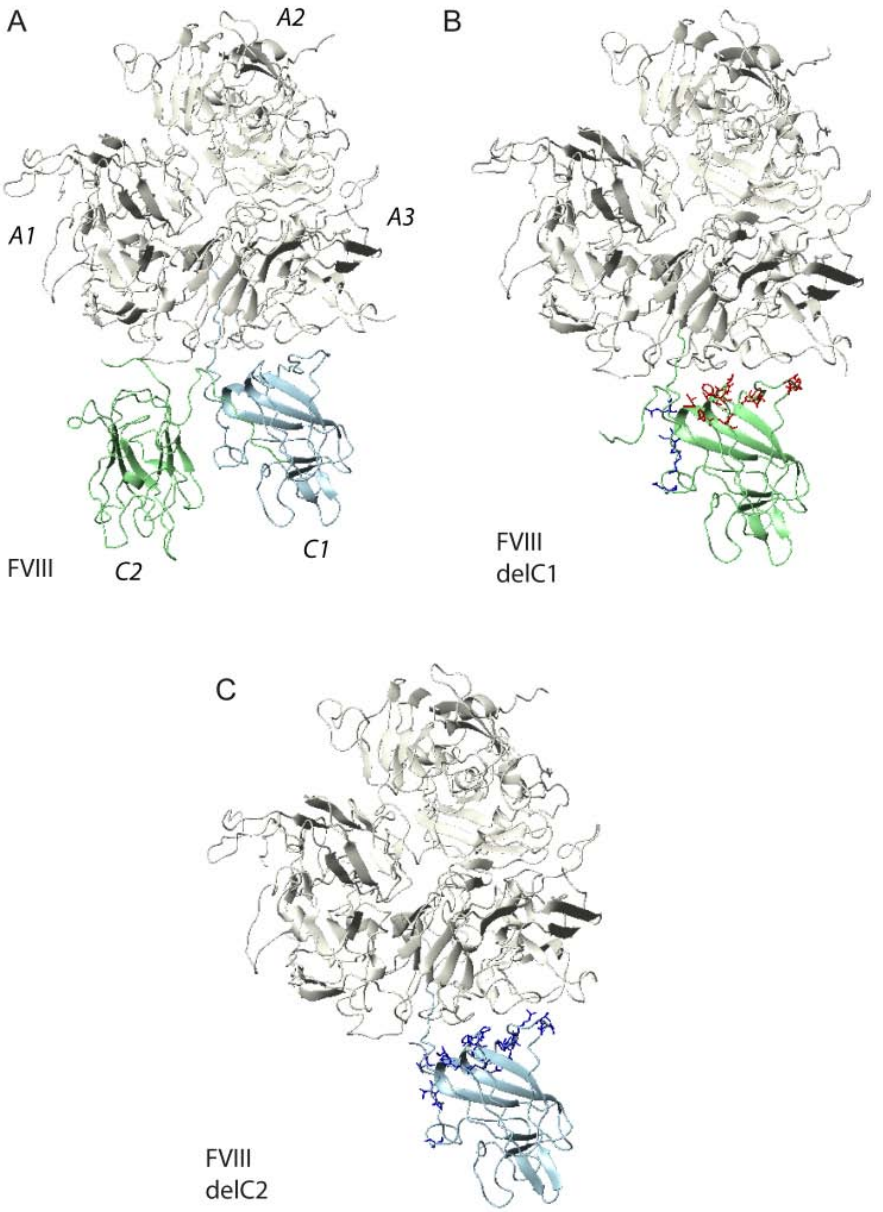

Figure 3. Models of FVIII C domain deletion variants. Normal FVIII (A), FVIIIdelC1 (B), and FVIIIdelC2 (C) were modeled to determine the effect of deletion of the C domains. The A domains are shown grey, C1 in blue and C2 in green. Residues interacting between A1-C2, A3-C1, and C1-C2[18,20] are depicted in blue when conserved, while non-conserved residues are shown in red. Models were based on the FVIII crystal structure (PDB code 2r7e)[18] and generated as described in the Methods section.

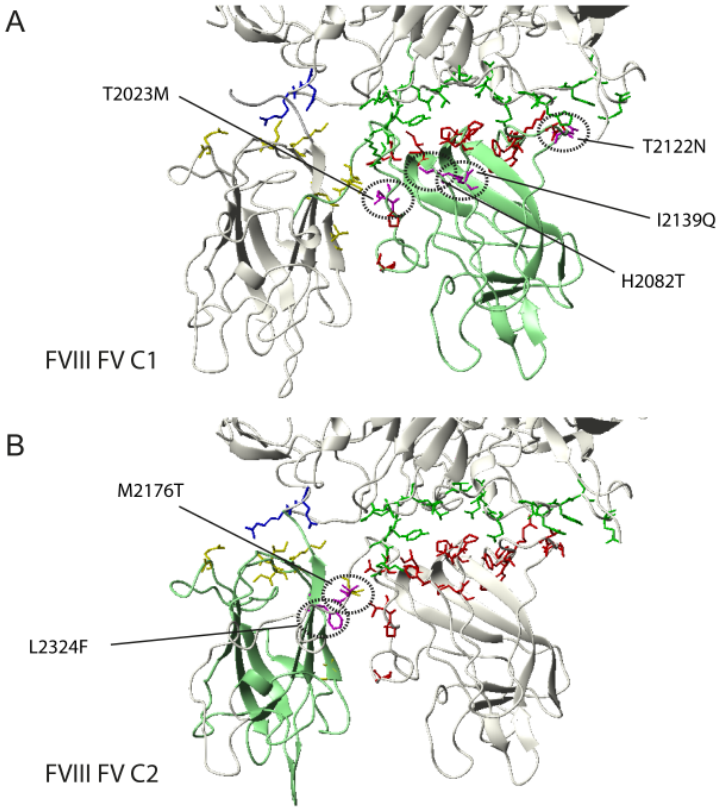

Figure 4. Models of FVIII FV C domain variants. Models of FVIII in which the C1 (A) or C2 (B) domain is exchanged for the corresponding domain of FV. The exchanged domain is shown in green. Interface residues of the A1 domain (blue), A3 domain (green), C1 domain (red), and C2 domain (yellow) are indicated.[18,20] Non-conserved residues on the interface are depicted in magenta and encircled. Models were based on the FVIII crystal structure (PDB code 2r7e)[18] and generated as described in the Methods section.

0.3±0.02 pmol/1×10^6 cells/72 h (mean±sd; n=3) were measured. We found that substitution of the C1 domain resulted in a dramatic decrease in co-localization of FVIII with VWF in WPBs (Figure 5A). Although some WPBs contained FVIII, most were negative for FVIII. This was in contrast to the mutant where the C2 domain was exchanged. The FVIII FV C2 variant sorted more efficiently to the WPBs than the FVIII FV C1 mutant, although not all transduced BOECs contained FVIII FV C2 in their WPBs (Figure 5A).

To rule out potential bias of disparities in expression levels, the sorting efficiency was quantified by subcellular fractionation. Besides that the FVIII FV C1 variant is poorly secreted, also the total intracellular FVIII/VWF ratio was very low compared to the other mutants (Table 2). While we could not detect FVIII FV C1 in WPB-containing fractions (Figure 5B and Table 2), it

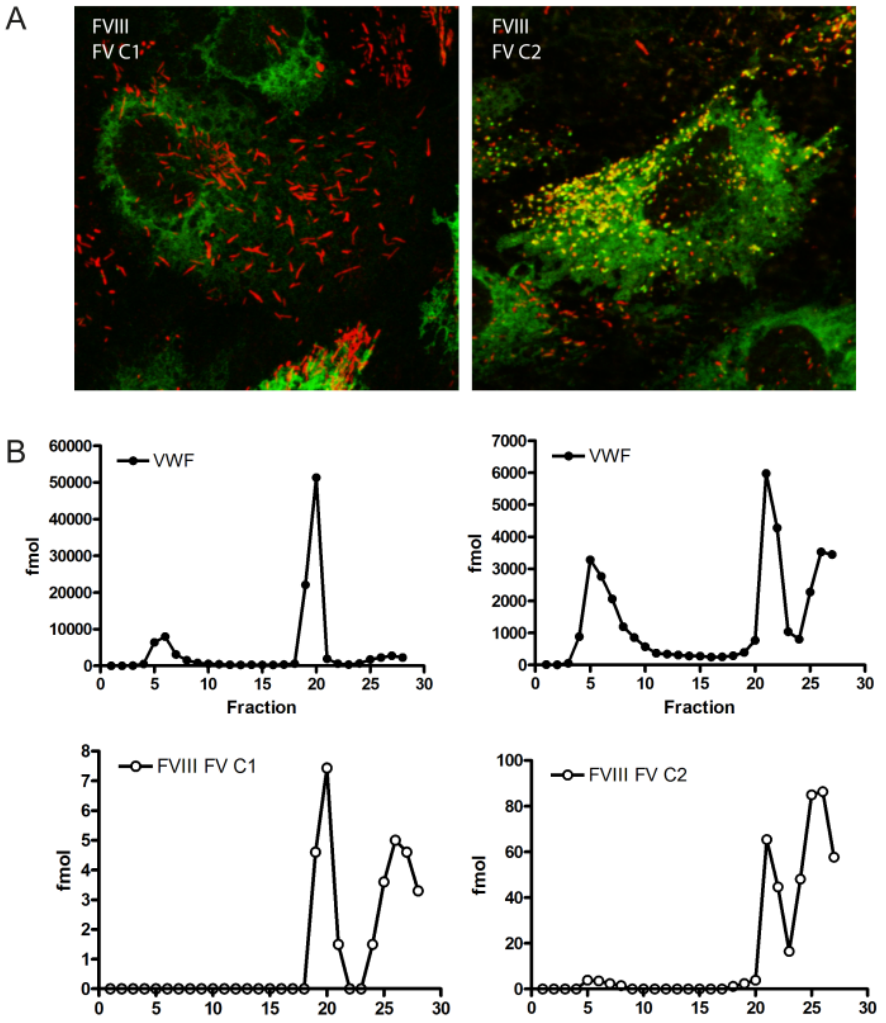

Figure 5. Intracellular localization of FVIII FV C1 and FVIII FV C2 variants in endothelial cells. (A) Confocal microscopy of BOECs expressing FVIII mutants shows that FVIII FV C1 (left panel; green) does not co-localize with VWF (red), while part of FVIII FV C2 (right panel; green) is stored in WPBs with VWF (red). (B) Sorting efficiency of FVIII FV C1 (left panel) and FVIII FV C2 (right panel) was determined by subcellular fractionation. VWF antigen (upper panels) and FVIII antigen (lower panels) were determined by ELISA. FVIII FV C1 was detected with antibody CLB-CAg9[17] and HRP-labeled CLB-CAg117[32], whereas FVIII FV C2 was detected using CLB-CAg12[33] and HRP-labeled CLB-CAg9[17].

is uncertain whether this caused by the exchange of the C1 domain or misfolding of the protein. Surprisingly, the sorting efficiency of FVIII FV C2 was only 2.7% (Table 2). Apparently, although some FVIII FV C2 targets to WPBs, only a small amount of protein is actually stored (Figure 5A-B). As such, this variant targets with the same low efficiency as FV (Figure 1B and Table 1). Our results show that exchanging the C2 domain of FVIII for the FV C2 domain results in a dramatic decrease in the WPB sorting efficiency.

Mutation of established phospholipid-binding residues does not affect FVIII sorting

Besides their structural similarities, FVIII and FV share the ability to assemble on negatively charged lipid membranes after activation.[21] In fact, FVIII and FV have similar affinities for phospholipid membranes, with a K_d of 3 nM and 4 nM respectively.[22,23] FV binds to phospholipid membranes via residues W2063/W2064 and Y1956/L1957.[22,24,25] W2063 and W2064 are located in a loop at the membrane binding surface of the C2 domain corresponding to a loop on FVIII that includes known lipid binding residues M2199/F2200.[26] The Y1956/L1957 residues in FV C1 domain are homologues to lipid binding residues K2092/F2093 in FVIII.[16] We mutated these lipid binding residues in the loop of the FVIII C1 domain to alanine, *i.e.* residues K2092 and F2093.[16,27] This FVIII variant was still able to sort to WPBs and additional mutation of lipid binding residues in the C2 domain (M2199A/F2200A/ L2251A/L2252A[26]) did not affect sorting of the FVIII variant to WPBs either (Figure 6). WPBs containing either one of two mutants were round and clearly positive for FVIII, confirming that these lipid-binding loops do not contribute to FVIII sorting to WPBs. These data combined with the low sorting efficiency of FV suggest that FVIII sorting to WPBs is not lipid-mediated.

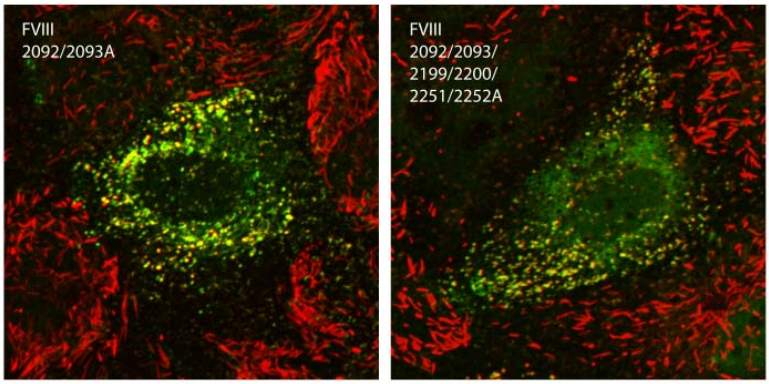

Figure 6. Sorting of two FVIII mutants to WPBs. FVIII 2092/2093A (left panel) and FVIII 2092/2093/2199/2200/2251/2252A (right panel) were expressed in BOECs by lentiviral transduction. Transduced BOECs were stained for FVIII with FITC-labeled CLB-CAg9[17] (green) and VWF with CLB-Rag20[17] (red). The intracellular localization of the FVIII mutants was visualized by confocal microscopy on a Zeiss LSM510 equipped with Plan NeoFluar 63x/1.4 Oil objective (Carl Zeiss, Heidelberg, Germany).

Discussion

It has been previously shown that FVIII expression in endothelial cells leads to sorting of FVIII to WPBs.[8-12] Nonetheless, the sorting mechanism behind FVIII sorting to WPBs is poorly understood. A recent study shows that 1-5% of total protein content in endothelial cells is missorted to WPBs by inefficient exclusion, irrespective of protein expression levels.[28] As FVIII sorts with approximately 20% sorting efficiency to WPBs, this suggests that FVIII is actively sorted to these organelles. On the other hand, we found a storage efficiency of 1-2% for FV, indicating that FV does not contain a specific sorting signal for storage in WPBs (Figure 1). It is unlikely that the absence of FV sorting to WPBs is the result of low intracellular FV content as we measured a 3-fold molar excess of FV compared to VWF within the endothelial cells, whereas roughly the opposite was true inside WPBs. Also expression levels indicated that FV was efficiently secreted by endothelial cells and was not retained in the ER. Therefore we conclude that the disparity in FV and FVIII sorting to WPBs is dependent on a FVIII-specific sorting signal.

In this study we show that both C domains contribute to sorting of FVIII to WPBs (Figure 2). Besides that deletion of the C1 domain reduces FVIII sorting from 23% to 13% (Figure 2 and Table 2), also exchanging the C1 domain for FV C1 completely prohibits WPB sorting (Figure 5 and Table 2). Although this points towards a role of the C1 domain in sorting to WPBs, there are several issues that greatly influence interpretation of the data. First, the normal domain orientation may be lost in the FVIIIdelC1 mutant, as the C2 domain is likely to be loosely attached at the position of the absent C1 domain (Figure 3B). Second, the low expression levels of the FVIII FV C1 variant may hinder detection of this mutant by confocal microscopy and subcellular fractionation (Figure 5 and Table 2). Finally, several critical residues in the C1-A3 interface are not conserved when the FV C1 domain is inserted (Figure 4). As the alignment of the C domains may be disrupted, the sorting to WPBs may be negatively influenced. To what extent the FVIII C1 domain is involved in sorting FVIII to WPBs therefore remains an open issue. In contrast, it seems likely that the domain orientation of both the FVIIIdelC2 and FVIII FV C2 mutants is conserved (Figures 3 and 4). Also the expression levels and total intracellular content of these mutants are comparable to normal FVIII (Table 2). This indicates that FVIIIdelC2 and FVIII FV C2 can be properly analyzed on their sorting efficiency. We show that not only deletion of the C2 domain leads to a radical decrease in the ability of FVIII to sort to WPBs (Figure 2 and Table 2) but also exchanging the FVIII C2 domain for the C2 domain of FV results in a significant reduction in the sorting efficiency (Figure 5 and Table 2). In fact, the 2.7% sorting efficiency of FVIII FV C2 is similar to FV (Figures 1B and 5B), which suggests that FVIII FV C2 is "missorted" to WPBs. Our data demonstrate that the FVIII C2 domain is essential for sorting FVIII to WPBs.

Two major pathways have been described to explain sorting of proteins to secretory vesicles, these being "sorting-for-entry" and "sorting-by-retention".[29,30] If FVIII sorts to WPBs by a "sorting-for-entry" interaction with VWF, this would mean that the intracellular ratio FVIII to VWF would reflect the percentage of FVIII stored in WPBs. Consider a situation in which *x* molecules of FVIII associate per *y* molecules VWF. Assuming that an excess of VWF is present

within the cell, a rise in the percentage of FVIII stored in WPBs may be expected when the intracellular ratio of FVIII to VWF would increase, and vice versa. However, in this study we found a similar percentage of FVIII stored in WPBs as in a previous study with GFP-tagged FVIII[9] (23% versus 15-22%, respectively), while the intracellular ratio of FVIII to VWF was considerably decreased (1:60 versus 1:10-1:23) due to higher VWF expression by this particular BOEC donor. Note that in all cases the percentage of FVIII stored in WPBs was comparable to the percentage of VWF inside WPBs. When we assume that on average WPB contain equal quantities of VWF, the percentage of VWF stored in WPBs can be used as an indication of the number of WPBs present in cells. In this case that would suggest that FVIII sorting to WPBs is more dependent on the number of WPBs present in endothelial cells than on intracellular VWF levels. Sorting-for-entry via an interaction with VWF would therefore be less likely.

Can we explain these observations with the "sorting-by-retention" model? The sorting-by-retention model proposes that a bulk flow of newly synthesized proteins is sorted to secretory vesicles, followed by active exclusion of soluble proteins that are destined for constitutive release. The sorting efficiency in this pathway would depend on the ability to bind proteins resident in the secretory vesicle.[30] This suggests that FVIII could still be retained in WPBs via an interaction with VWF. Thus, instead of sorting *x* molecules of FVIII per *y* molecules VWF, we would retain *x* molecules of FVIII per *y* molecules VWF present in WPBs. As we assume that WPBs contain similar amounts of VWF, an equal amount of FVIII would be retained per WPB. Therefore FVIII sorting to WPBs would be dependent on the number of WPBs in endothelial cells, even though FVIII is retained via an interaction with VWF. This is in agreement with our finding that the percentage of FVIII in WPBs is independent on the total intracellular FVIII/VWF ratio. We therefore propose that FVIII enters WPB via bulk flow, which then is retained according to the "sorting-for-retention" model via a yet unknown interaction of the C domains with VWF.

The apparent dependency of FVIII sorting on the percentage of VWF in WPBs is in agreement with our observations on sorting of FV (Figure 1). The low sorting efficiency of FV to WPBs might be due to a lack of interaction with VWF. In contrast to FVIII, FV does not circulate in complex with VWF, which makes FV-VWF complex assembly very unlikely. The FVIII C1 and C2 domains contain multiple residues contributing to extracellular FVIII-VWF complex formation, including residues I2098, S2119, N2129, R2150, P2053 in the C1 domain and W2229, Q2246, A2201, M2199, F2200, L2251 and L2252 in the C2 domain.[26,31] However, we have previously determined that mutation of S2119, R2150 or A2201 did not result in reduced sorting even though these mutants displayed a strong decrease in VWF binding (Chapter 3). In this paper we show that mutation of residues M2199, F2200, L2251, and L2252 also does not affect sorting of FVIII to WPBs (Figure 6). Further research is needed to determine the contribution of individual amino acid residues in the FVIII C domains, and in particular in the C2 domain, to WPB sorting. By focussing on FVIII residues that are different in FV, the structural requirements for FVIII sorting to WPBs might be identified.

Acknowledgements

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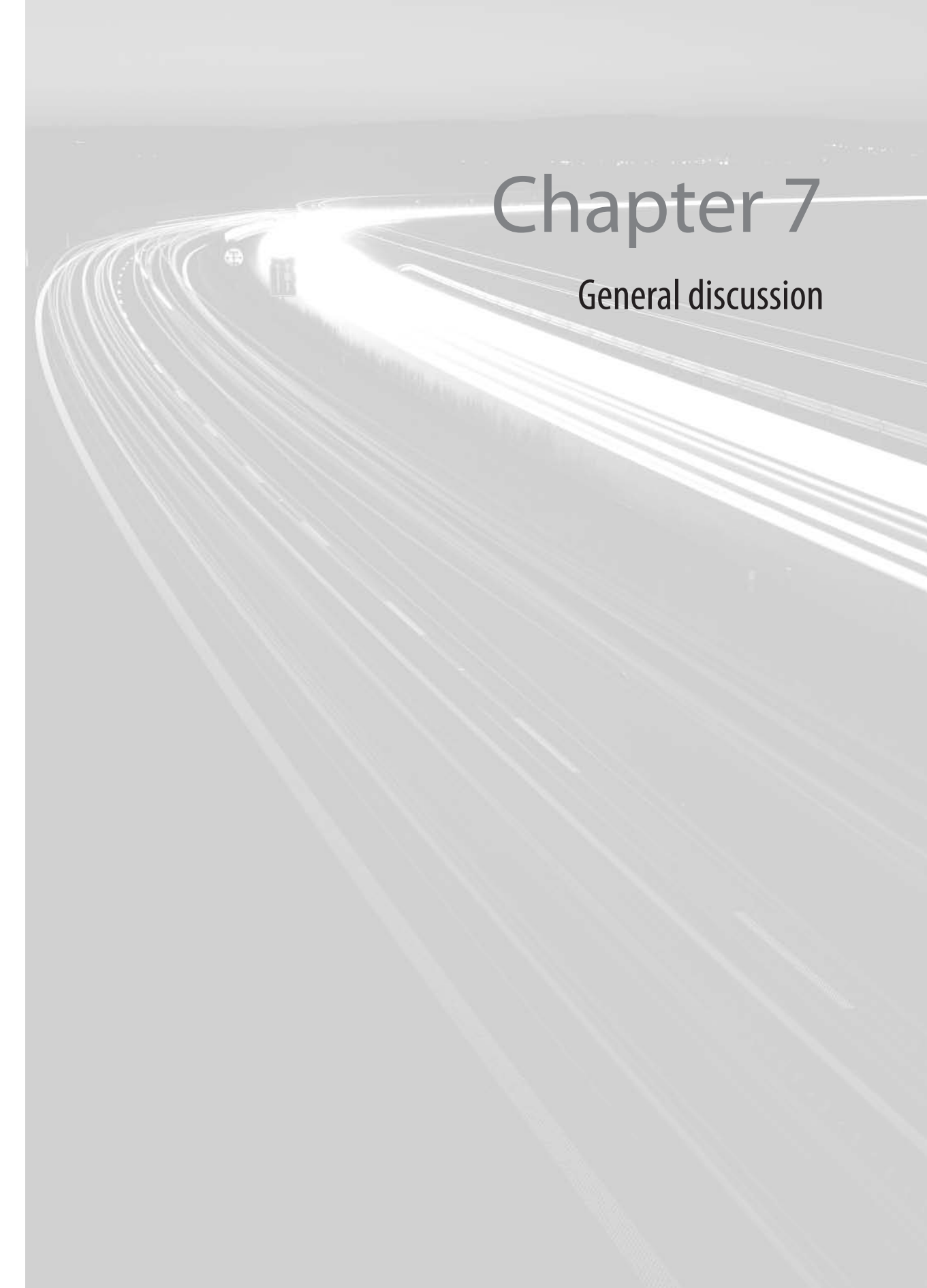

Chapter 7

General discussion

An attractive vehicle for cellular delivery of factor VIII (FVIII) is represented by endothelial cells. Endothelial cells are one of the two cell types that store von Willebrand factor (VWF), the natural carrier protein of FVIII. Expression of FVIII in endothelial cells leads to co-storage of FVIII with VWF in secretory Weibel-Palade bodies (WPBs).[1,2] Exocytosis of WPBs therefore potentially releases FVIII in complex with VWF. Direct release of FVIII-VWF complex from endothelial cells may offer secretion of high FVIII levels with prolonged circulation time, which could be essential for successful gene therapy. So far FVIII gene therapy trials have failed to achieve sustained FVIII levels.[3-6] This could be the result of targeting the wrong cell for FVIII expression, as in most studies hepatocytes were targeted. Although the applicability of FVIII-expressing endothelial cells has been studied in mice models[7,8], little attention has been paid to FVIII-VWF co-delivery on a cellular level. Thus several key questions remain unanswered. These include: how much FVIII can be released from FVIII-expressing endothelial cells? Is all synthesized FVIII stored in WPBs? Are FVIII and VWF indeed released in complex? Are FVIII and VWF functionally active upon release from FVIII-expressing endothelial cells? How does FVIII traffic to WPBs and, if necessary, can we improve the amount of FVIII stored in WPBs? Knowledge of these issues may help to achieve successful gene therapy with delivery of FVIII by endothelial cells. Moreover, understanding the molecular interactions occurring in FVIII-expressing endothelial cells can shed light on results obtained from animal experiments. In this thesis we addressed molecular details of FVIII-VWF co-expression to determine whether endothelial cells indeed are suitable for FVIII delivery.

Benefits of cellular FVIII delivery

The majority of research on FVIII gene therapy has focused on systemic delivery of viral vectors, often targeted to hepatocytes.[9] Unfortunately, systemic delivery of viral vectors in general suffers from vector-mediated toxicity and induction of the immune system.[10] These issues can be circumvented by using a cellular delivery device, especially when autologous cells are used. Besides that this way no viral vector is introduced *in vivo,* also an immune response to autologous cells is less likely to occur. Nonetheless, the major bottleneck for all FVIII gene therapy approaches has been formation of neutralizing antibodies to FVIII.[3,9] While systemic administration of viral vectors results in high FVIII levels in hemophilic mice, FVIII levels rapidly decline due to formation of neutralizing antibodies.[9,11,12] Even though inhibitory antibodies are less frequently found in hemophilic dogs, FVIII levels in dogs are low and mostly transient.[13] Intracellular FVIII storage pools are not accessible for neutralizing antibodies and therefore on-demand release of stored FVIII may arrest bleeding before circulating inhibitory antibodies associate with FVIII. Furthermore, it has been suggested that FVIII in complex with VWF is less immunogenic.[14] This provides cells that produce and store VWF with an additional advantage over other cell-based delivery devices. Thus the best suitable cell for FVIII delivery would be an autologous, VWF-producing cell that releases FVIII on-demand.

FVIII-VWF co-delivery in small animals

The benefit of delivering FVIII in complex with its carrier protein VWF is illustrated by *in vivo*

experiments with transgenic FVIII-KO mice that only express FVIII in megakaryocytes.[15,16] While plasma FVIII cannot be detected, the quantity of FVIII stored in platelet α-granules constitutes approximately 1% of normal FVIII plasma content.[17] Also transduction of bone marrow with a lentiviral vector followed by transplantation of these cells in hemophilic recipient mice leads to sustained FVIII expression without inhibitor formation.[18,19] Strikingly, in the presence of inhibitory antibodies to FVIII, transgenic mice that co-store FVIII with VWF in platelet α-granules have a better change to survive a tail clip test compared to mice that do not contain VWF in their platelets.[16] Interesting to note is that FIX stored in α-granules of platelets is not effective in the presence of neutralizing antibodies to FIX.[20] Therefore, release of a protein from storage pool alone is not enough to circumvent neutralizing antibodies. This supports the conclusion that FVIII-VWF complex delivery is superior over FVIII expression alone, especially in the presence of inhibitory antibodies.

Although the data mentioned in the previous paragraph underline the need of FVIII-VWF co-delivery, platelets may not be the ideal cell type for FVIII delivery for several reasons. First, results of platelet-derived FVIII treatment differ with the bleeding model used. While $FeCl_3$-induced injury and tail clip survival tests demonstrate complete correction, whole blood clotting time and cuticule bleeding time are only partially corrected.[15,16] Second, a major drawback of FVIII delivery by platelets is an association with clot embolism. Neyman and co-workers[21] showed that differences in time and place of FVIII delivery by platelets alter fibrin and platelet accumulation within clots. This leads to an increase in the size and number of emboli.[21] Third, thrombocytotic stimuli that increase the platelet count do not affect the amount of FVIII synthesized by megakaryocytes. While the total systemic FVIII content remains the same, this results in a reduction of the intracellular FVIII content per platelet. This completely reverses the phenotypic bleeding correction when TPO-treated hemophilic mice are subjected to a tail clip survival test.[22] Thrombocytotic stimuli such as inflammatory responses often occurring during bleeding episodes, may therefore lead to less efficient bleeding arrest by platelet-derived FVIII. Finally, a key problem of FVIII expression in platelets is acquiring autologous megakaryocytes. Megakaryocytes can be obtained from hematopoietic stem cells in bone marrow and modified to express FVIII[19]. However, this invasive procedure poses a heavy burden on patients compared to the current therapy consisting of protein replacement. Evidently, the issues outlined above limit the feasibility of platelets as cellular delivery device for FVIII.

Besides platelets also endothelial cells contain a storage pool of VWF. Recently, Xu and co-workers reported that induced pluripotent stem (iPS) cells differentiated to the endothelial lineage secrete endogenous FVIII.[23] Moreover, transplantation of these cells in hemophilic mice leads to FVIII levels that are 8-12% of normal. Interestingly, one year after transplantation mice treated with differentiated iPS cells have FVIII levels normal or higher than those of wild type mice.[24] Whether or not iPS cells differentiated to the endothelial lineage express VWF was unfortunately not stated. Although these are promising results, the applicability at this point is limited by the laborious generation of iPS cells. iPS cells used by Xu *et al.* are formed from mouse skin fibroblasts, and as such easy to obtain. However, to generate iPS cells from skin fibroblast a total of four transcription factors have to be inserted by retroviral

transduction.[23] In addition, iPS cells then still have to be differentiated to endothelial cells.

In contrast to iPS cells and megakaryocytes, autologous blood outgrowth endothelial cells (BOECs) are easy to obtain, as they can be readily isolated from small volumes of peripheral blood. Moreover, direct infusion of BOECs expressing FVIII in immunodeficient mice leads to sustained FVIII expression.[7] After 156 days all treated mice have FVIII levels nearing or exceeding the physiological concentration in wild type mice. Surprisingly, FVIII levels increase over time, which suggests that BOECs expand *in vivo*.[7] An alternative for direct infusion of cells in circulation is administering FVIII-expressing BOECs subcutaneous in a Matrigel scaffold so transduced cells are contained in a restricted area.[8] In this case, FVIII expression lasted 12 weeks with on average 2% of normal FVIII levels. By changing the promoter for FVIII expression to an endothelial-specific promoter, FVIII levels can be detected for at least 27 weeks. Unfortunately, after 30 weeks no FVIII is detected anymore, which is accompanied by disappearance of the Matrigel implant. Therefore, breakdown of the Matrigel scaffold is probably responsible for reduction of FVIII levels.[8] Recently Shi, *et al*.[25] generated transgenic mice that express FVIII in endothelial cells. Transgenic mice have normal plasma levels (in contrast to transgenic mice expressing FVIII in platelets) and have a storage pool of FVIII that can be released upon epinephrine treatment. However, in the presence of inhibitors the survival rate is not higher than immunized wild type mice.[25] It is worth mentioning that the transgenic mice did not receive epinephrine prior to or following tail clipping, so FVIII pools stored with VWF in endothelial cells may not have been released. In conclusion, endothelial cells provide normal circulating FVIII levels and complete tail clip survival in the absence of inhibitory antibodies but are possibly less effective for the treatment of hemophilic patients that have neutralizing antibodies to FVIII.

FVIII-VWF delivery by endothelial cells: a cellular perspective

Can we explain the *in vivo* observations discussed above with the results described in this thesis? We found that while BOECs transduced with a lentiviral vector encoding FVIII express high levels of FVIII, only a small amount of FVIII is released upon stimulation of WPB exocytosis (Chapter 2). Quantification of the amount of FVIII stored in WPBs reveals that approximately 20% of total intracellular FVIII is stored with VWF in WPBs. Therefore most FVIII is constitutively released and thus probably not in complex with VWF. This eliminates for a large part the potential benefits of FVIII-VWF complex secretion. Although BOECs can constitutively release enough FVIII to correct the hemophilic phenotype in mice, the amount of FVIII stored in WPBs alone may not be sufficient when constitutively secreted FVIII is removed from circulation by neutralizing antibodies. This is not unlikely as reduction of the intracellular FVIII content in platelets by an increase in platelet count also leads to an inability to arrest bleeding.[22] Apparently a threshold of intracellular stored FVIII is needed to efficiently arrest bleeding. Therefore it may be of great importance to redirect constitutively secreted FVIII to the secretory pathway.

To increase the quantity of FVIII stored with VWF, the sorting mechanism for FVIII trafficking to WPBs needs to be elucidated. In chapter 6 we determined that the C domains of

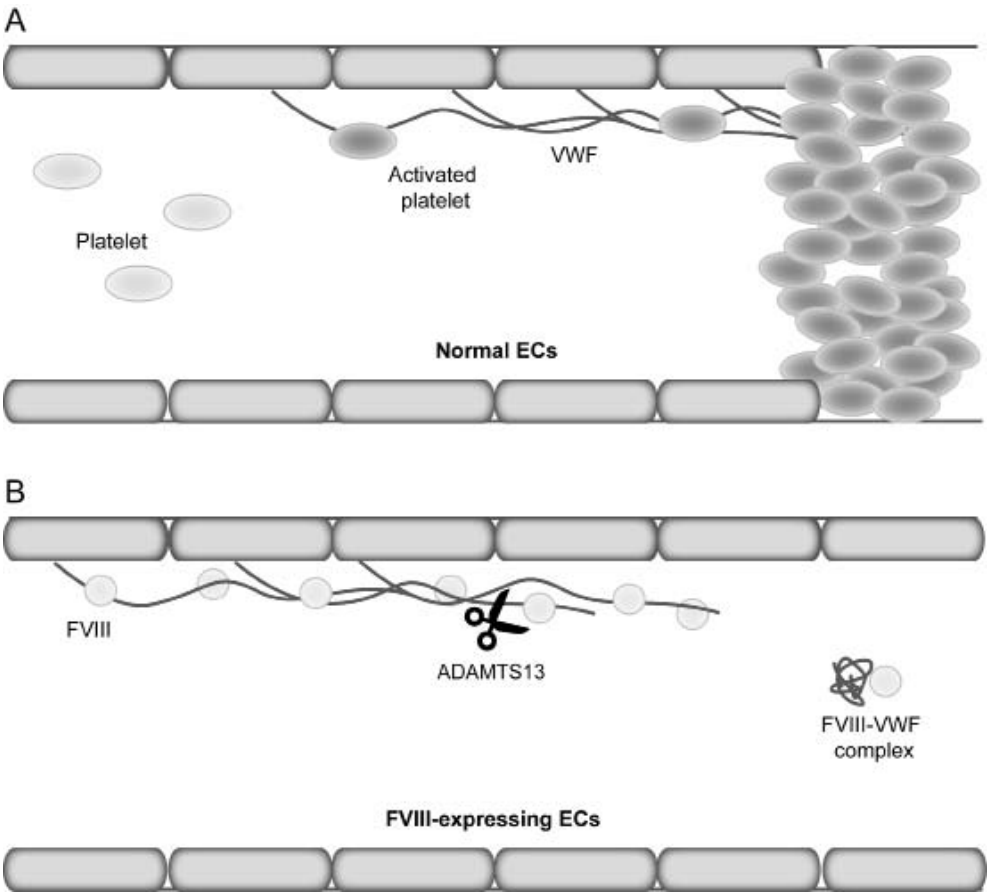

Figure 1. Model for the function of VWF released by FVIII-expressing endothelial cells. VWF released by normal endothelial cells (A) is able to recruit platelets to sites of vascular damage. (B) FVIII on VWF strings released by FVIII-expressing endothelial cells prevents platelet adhesion and enhances cleavage of VWF strings by ADAMTS13. This results in release of FVIII-VWF complex in circulation.

FVIII contribute to trafficking of FVIII to WPBs. Although the extracellular high-affinity binding sites of FVIII for the D'-D3 region on VWF are not responsible for targeting FVIII to WPBs (Chapter 3 and ref [1]), it is still likely that VWF serves as the sorting chaperone for FVIII. Not only does FVIII expression disrupt VWF tubules in WPBs (Chapter 4) but also does endogenously synthesized FVIII bind better to VWF strings compared to exogenously added FVIII (Chapter 5). These data prove that intracellular interactions between FVIII and VWF indeed occur. Unfortunately, at this point the exact residues involved in the intracellular interaction of FVIII with VWF have not been identified. Clearly intracellular and extracellular FVIII-VWF interactions differ greatly, as disruption of VWF tubules, disturbance of ultra-large VWF multimers, reduction of platelet adhesion to VWF strings, binding of FVIII to VWF strings and FVIII sorting to WPBs are all independent of the critical extracellular VWF binding site Y1680 (Chapter 3-5). In chapter 3 we demonstrate that extracellular binding of FVIII Y1680F to VWF is enhanced at low pH. These

data suggest that the high-affinity interaction mediated via Y1680 has a pH optimum of 6.7, whereas a second, low-affinity interaction has a pH optimum of 5.5. Since the pH inside WPBs is estimated to be 5.5[26], intracellular FVIII-VWF interactions are possibly also driven by this low-affinity binding site. As intracellular FVIII and VWF levels may be more concentrated in WPBs than in solution, this undisclosed low-affinity interaction could be enough for FVIII to associate with VWF inside WPBs. The prominent reduction in platelet binding of VWF strings released by both FVIII- and FVIII Y1680F-expressing cells (Chapter 4) suggests that the VWF A1 domain (responsible for binding of platelets) may carry this low-affinity binding site for FVIII.

FVIII disrupts tubular storage of VWF: clinical implications?

A striking feature of FVIII storage in WPBs is that FVIII expression disrupts VWF tubule formation to such an extent that FVIII-containing WPBs become round.[2,27,28] We demonstrated in chapter

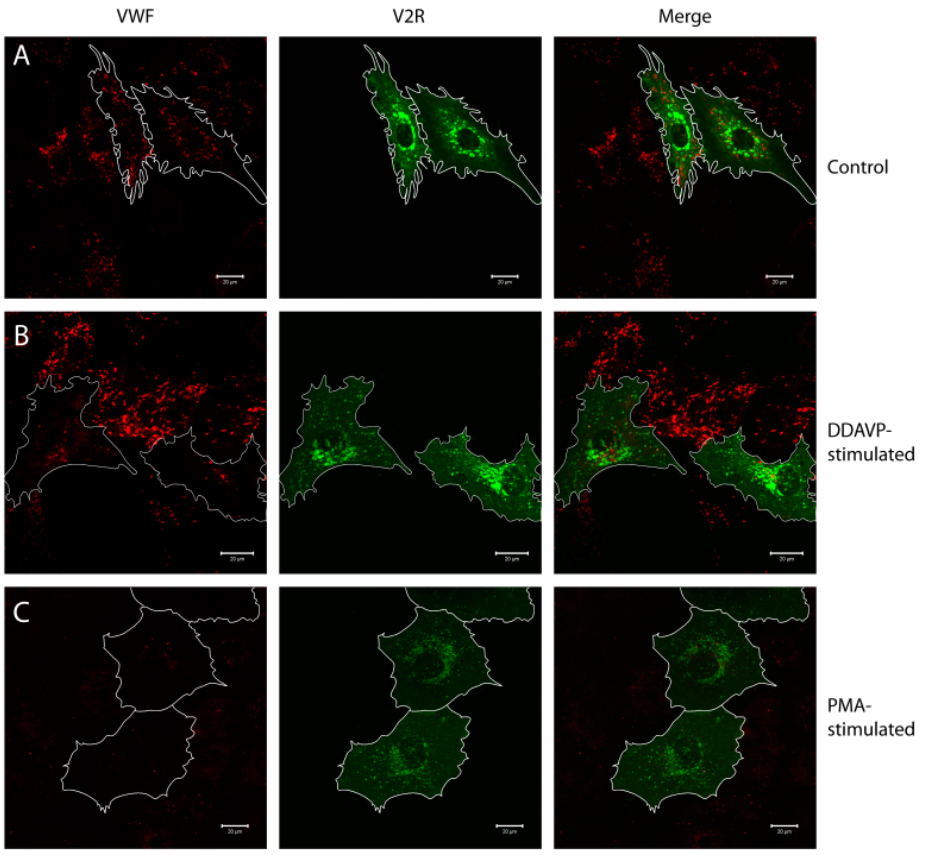

Figure 2. WPB exocytosis from V2R-transduced endothelial cells. Confluent V2RGFP-transduced BOECs were incubated for 1 hour with serum-free (SF) medium (50% RPMI-1640 and 50% M199-Hepes) supplemented with 1% (v/v) HSA (Cealb, Sanquin, Amsterdam, the Netherlands), 0.3 mg/ml L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin) (A), SF medium supplemented with 1 μM DDAVP and 100 μM IBMX (B) or supplemented with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) (C). Cells were fixed with 3.7% PFA and stained for VWF (red) using CLB-RAg20[33].

4 that FVIII-containing WPBs have short, disorganized VWF tubules. Despite the absence of long VWF tubules, VWF strings of normal length are released. However, these strings recruit fewer platelets than strings originating from non-transduced BOECs. The reduced platelet binding to strings released from FVIII-containing WPBs apparently positions FVIII as an antagonist of primary hemostasis. An anticoagulant role for FVIII has also been recently proposed by Skipwith and co-workers[29], who found that FVIII acts as a cofactor for cleavage of VWF strings by the metalloprotease ADAMTS13. This raises the possibility that FVIII storage with VWF alters the role of VWF in coagulation. The primary goal of VWF release from FVIII-expressing endothelial cells would not be to recruit platelets (Figure 1A) but to remain associated with FVIII (Figure 1B). FVIII storage would prevent platelet adhesion to secreted VWF strings, while strings covered with FVIII are quickly cleaved by ADAMTS13, thereby releasing FVIII-VWF complexes in circulation.

FVIII binds to VWF strings: clinical implications?

An intriguing observation has been that FVIII binds to VWF strings released by endothelial cells (Chapter 5). Even more surprising is that despite the absence of the extracellular high-affinity binding site for VWF, FVIII Y1680F remains associated with VWF strings after release from endothelial cells. The fluorescence FVIII intensity of 'endogenously' secreted FVIII Y1680F on VWF strings is similar to normal FVIII. In contrast, binding of exogenously added FVIII Y1680F is reduced. The observed difference in binding of endogenous and exogenous FVIII Y1680F suggests that binding of exogenously added FVIII is more dependent on tyrosine 1680 binding to VWF, while binding of endogenous FVIII could be mediated through a different interaction. Domains that play a minor role in extracellular FVIII-VWF complex assembly may contribute considerably in intracellular binding of FVIII to VWF. Moreover, FVIII pre-incubated with VWF and subsequently perfused over endothelial cells is able to bind to VWF strings (Chapter 5). This indicates that also *in vivo* circulating FVIII may bind to VWF strings released at sites of vascular damage. The current paradigm is that FVIII, to participate in coagulation, is first cleaved by thrombin, then dissociates from VWF and finally binds to exposed PS on activated platelets. Cleavage of FVIII by thrombin is known to reduce the affinity of FVIII for VWF and therefore may lead to dissociation of the complex.[30] While this appears plausible there is one major problem with this concept: FVIII has to be quickly captured from the circulation. By the time FVIII is activated by thrombin, the FVIII-VWF complex may have already passed the site of injury. An attractive alternative would be that FVIII is first captured from circulation and then activated by thrombin. Binding of FVIII to VWF strings released at sites of injury may give a local rise of FVIII without the need of activation by thrombin. Once bound to VWF strings, cleavage by thrombin may release FVIII from the strings to contribute to localized blood coagulation.

Restoration of DDAVP-responsiveness of BOEC

We have shown in chapter 2 that WPB exocytosis can be triggered by treating endothelial cells with thrombin, phorbol 12-myristate 13-acetate (PMA) and epinephrine. However, these

compounds are not desirable for *in vivo* use. For adequate FVIII delivery by BOECs, a physiological method to release FVIII from WPBs has to be developed. Desmopressin (DDAVP) is widely used in the clinic to treat patients with von Willebrand's disease (VWD).[31] DDAVP is a selective agonist for the vasopressin-2 receptor (V2R), and is known to cause a rapid increase in FVIII and VWF levels.[32] Because of the clinical experience with DDAVP, *in vivo* induction of WPB exocytosis with DDAVP is the preferred method to release FVIII. In our own experiments where non-transduced BOECs are treated with DDAVP, numerous WPBs can still be detected by confocal microscopy. In addition, only a slight increase in VWF concentration in conditioned medium is measured (1.5-fold increase, $p>0.05$, data not shown). The low VWF secretion by BOECs upon stimulation with DDAVP indicates that the V2 receptor is not or barely expressed on BOECs, or is lost during culture. These findings are in agreement with previously published work by Kaufmann and co-workers[33], who found that HUVEC do not respond to stimulation with DDAVP. However, transfection of HUVECs with the V2 receptor results in DDAVP-inducible exocytosis of WPBs.[33] We have also addressed the possibility of V2R over-expression in BOECs to restore DDAVP-inducible WPB exocytosis. Preliminary data shows that in BOECs transduced with a GFP-tagged V2R construct V2R-positive punctuations can be detected throughout the cells (Figure 2A). When treated with DDAVP only those cells that are positive for V2R release their WPBs (Figure 2B), whereas PMA induces exocytosis of WPB in all cells (Figure 2C). Unfortunately, when VWF release is quantified by ELISA only a limited (though statistically significant) increase of VWF can be measured (Figure 3). This is probably caused by the low number of V2R-positive cells in the cell population due to inefficient transduction. As figure 1B shows that V2R-positive cells respond well to DDAVP-stimulation, it is to be expected that VWF release will increase when the cell population is more positive for V2R. Therefore over-expression of V2R on FVIII-transduced BOECs may be a suitable approach to generate a DDAVP-inducible release-on-demand device.

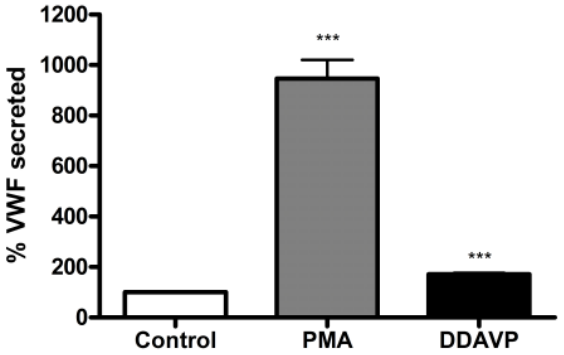

Figure 3. Quantification of VWF release from V2R-transduced BOECs. V2RGFP-expressing BOECs were incubation with SF medium containing 50 ng/ml PMA or 1 μM DDAVP plus 100 μM IBMX. VWF release was quantified using an ELISA with monoclonal CLB-CAg20[33] as primary antibody and HRP-labeled polyclonal rabbit anti-human VWF (DAKO, Glostrup, Denmark) as secondary antibody. Normal human pooled

Potential of BOECs as a cellular FVIII delivery device

For various reasons outlined in the preceding paragraphs, FVIII delivery by autologous cells that store FVIII with VWF inside secretory vesicles would be preferred. Autologous BOECs comprise a promising cell type as these cells synthesize and store VWF in WPBs, and can be easily obtained from peripheral blood. Moreover, large quantities of FVIII are secreted by BOECs, which does not only result in clinically relevant plasma FVIII levels but also in phenotypic correction of hemophilia A in mice.[7,8] However, the largest part of synthesized FVIII (~80%) is not co-stored with VWF in WPBs but is released via the constitutive pathway (Chapter 2). This may explain why in the presence of neutralizing antibodies to FVIII the efficacy of endothelial-derived FVIII is decreased when release of the intracellular storage pool is not stimulated.[25] Presumably, FVIII is released from WPBs in complex with VWF as exocytosis of WPB content under flow results in strong association of FVIII with VWF strings (Chapter 5). Although VWF released from FVIII-containing WPBs is less efficient in recruiting platelets (Chapter 4), this phenotype is most likely reversed when FVIII dissociates from VWF strings upon activation by thrombin. If the targeting efficiency of FVIII to WPBs can be increased, basal release (Chapter 2) of FVIII-VWF complex from WPBs may be beneficial for hemophilia A therapy, especially when release of additional FVIII-VWF complex can be triggered by DDAVP treatment. Future research should therefore focus on increasing the targeting efficiency of FVIII to WPBs and restoring the DDAVP-responsiveness of FVIII-expressing BOECs. Furthermore, it would be interesting to know which molecular interactions play a role in the intracellular assembly of the FVIII-VWF complex. Our studies suggest that this could encompass both C domains of FVIII and the VWF A1 domain. Based on the results obtained in chapter 3, mapping of FVIII-VWF contact sites at low pH may be needed to resolve this issue.

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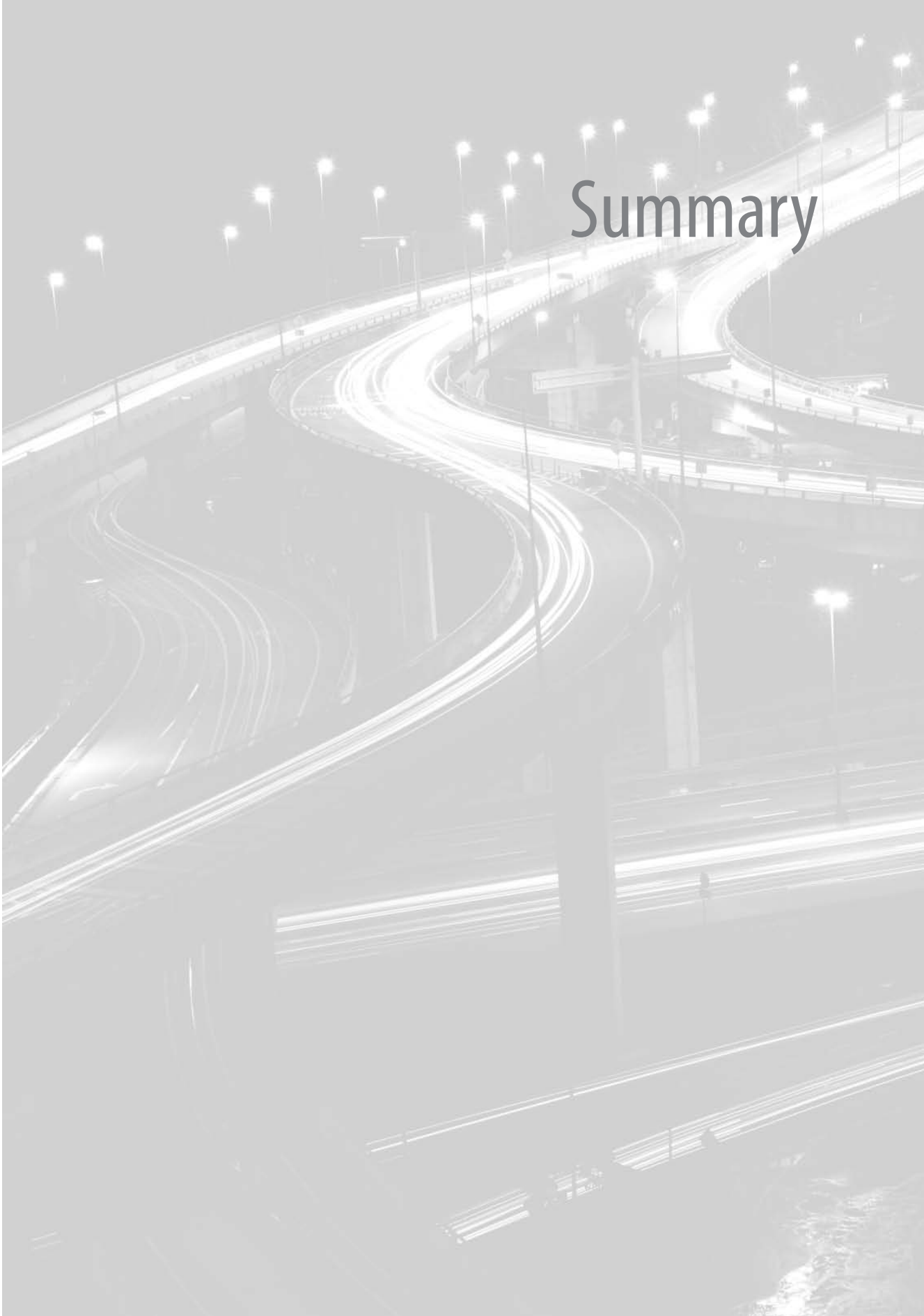


Summary

A defect in coagulation factor VIII (FVIII) results in the inherited bleeding disorder hemophilia A. Current treatment of hemophilia A is hampered by the need of frequent administration of costly FVIII products. Therefore gene therapy is an attractive alternative for protein replacement to treat hemophilia A patients. Recent insights have suggested that it may be beneficial to express FVIII in cells that also secrete its natural carrier protein von Willebrand factor (VWF), *i.e.* platelets and endothelial cells. In this thesis our aim was to explore the potential of endothelial cells as a cellular release-on-demand device for co-delivery of FVIII and VWF.

An overview of the current knowledge on FVIII and VWF biosynthesis, storage and molecular interactions is given in **chapter 1**. While the liver is considered to be the main site of FVIII synthesize, increasing evidence suggests that endothelial cells offer an alternative source for FVIII. FVIII expressed by endothelial cells is stored with VWF in secretory Weibel-Palade bodies (WPBs), which could provide a storage pool for direct release of FVIII-VWF complex. As FVIII in complex with VWF has prolonged circulation time, is protected from proteolytic inactivation and immune response, release of FVIII-VWF complex from endothelial cells is considered to be superior over release of FVIII from hepatocytes or other cell types that do not synthesize VWF.

In **chapter 2** we demonstrate that blood outgrowth endothelial cells (BOECs) respond robustly to various stimuli with release of VWF stored in WPBs. BOECs transduced with a lentiviral vector encoding FVIII are capable of long-term production of high FVIII levels. A substantial part of FVIII synthesized by endothelial cells is stored with VWF in WPBs, and can be released upon stimulation of WPB exocytosis. Using confocal microscopy we observed that the appearance of FVIII-containing WPBs was round as opposed to the typical elongated shape of normal WPBs.

The role of VWF as a sorting chaperone for FVIII is addressed in **chapter 3**. We studied the sorting ability of FVIII variants displaying reduced VWF interaction. While the FVIII mutants had moderate (R2150H, Del2201, P2300S) or severe (Y1680F, S2119Y) VWF binding defects, expression studies in HEK293 cells and BOECs, revealed that all variants were present in (pseudo-)WPBs. Moreover, quantification of the sorting efficiency demonstrated that the relative amount of FVIII storage was independent of the mutations. Our data suggest that FVIII sorting to WPBs is not affected by a reduced extracellular FVIII-VWF interaction.

The change in WPB morphology caused by FVIII expression is studied in more detail in **chapter 4**. We found that FVIII expression prohibits the formation of long VWF tubules that normally determine the elongated shape of WPBs. VWF tubules form strings that can recruit platelets once VWF is released from WPBs. Despite the absence of long tubules, release of WPBs from BOECs expressing normal FVIII or FVIII Y1680F resulted in VWF strings that were equally long as those from non-transduced BOECs. However, the released strings had largely lost their ability to recruit platelets. These findings suggest that an intracellular interaction of FVIII with VWF during formation of WPBs impairs recruitment of platelets.

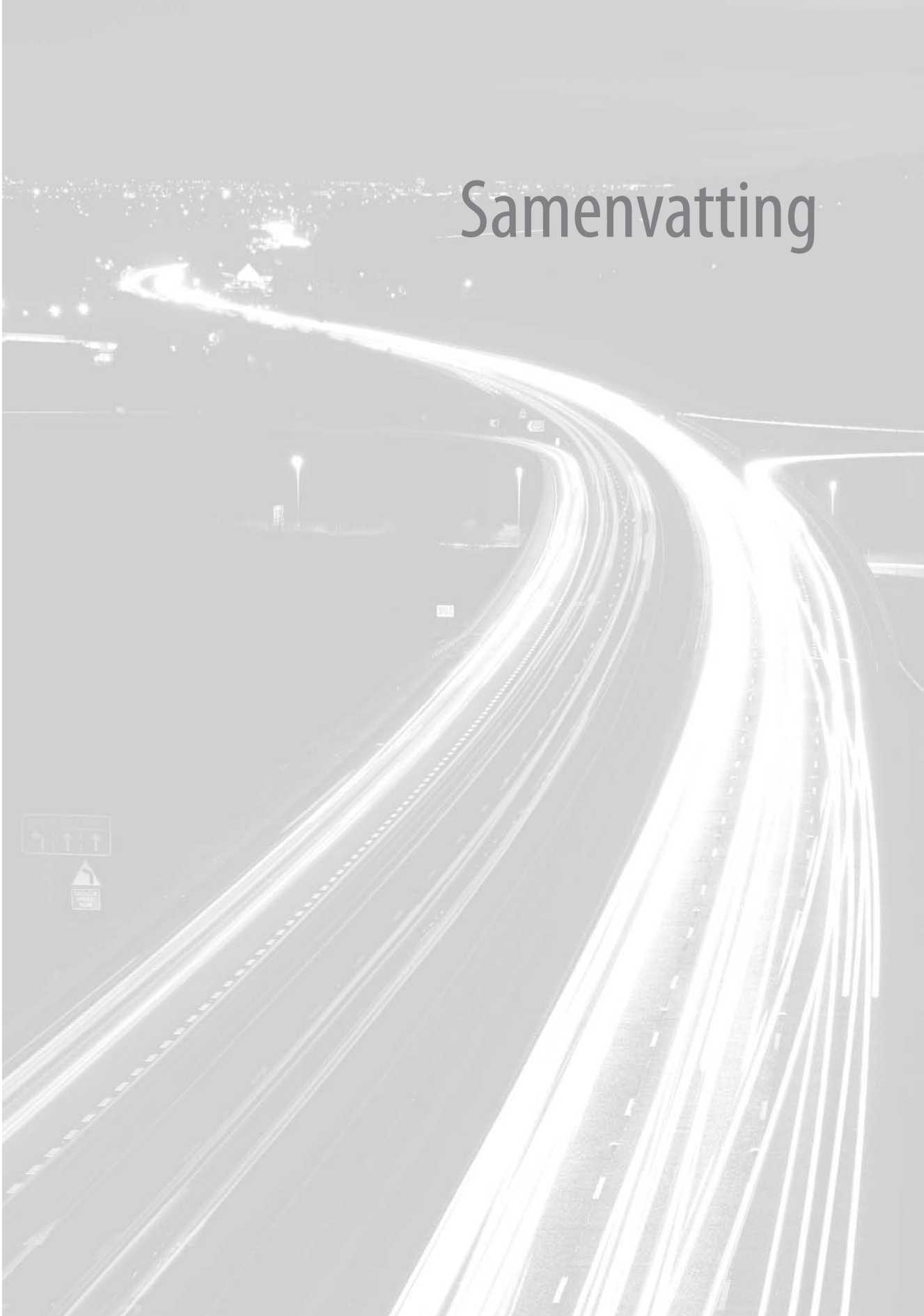
The effect of FVIII expression on VWF stored in WPBs implies that FVIII and VWF associate with each other inside WPBs. In **chapter 5** we studied whether or not WPB-derived FVIII and VWF remain associated after release from WPBs. We found that FVIII co-stored with

VWF in WPBs binds to secreted VWF strings without a need of the extracellular high-affinity VWF binding site Y1680. Exogenously added normal FVIII binds VWF strings released from non-transduced BOECs, whereas exogenous FVIII Y1680F does not associate with VWF strings to the same degree. Apparently, intracellular and extracellular FVIII-VWF complex assembly differs with regard to the contribution of the Y1680 residue in VWF binding.

We further address the intracellular association of FVIII with VWF in **chapter 6**. As factor V (FV) and FVIII are structurally highly homologous, we studied the possibility that FV may also sort to WPBs. Because we found that the efficiency of FV sorting to WPBs is very low, it seems likely that an interaction with VWF is needed for sorting to WPBs. We addressed whether the C domains are essential for sorting to WPBs by deletion of these domains, and exchanging C domains of FVIII for corresponding domains of FV. Our results demonstrate that FVIII sorting to WPBs is driven by FVIII-specific structural elements in both C domains, but in particular the C2 domain.

Finally, in **chapter 7** we discuss our findings in view of the applicability of BOECs as a delivery device for FVIII. In addition, we address other cellular FVIII delivery devices that have been described in literature.

Samenvatting



Een defect in stollingseiwit factor VIII (FVIII) leidt tot de erfelijke bloedingsziekte hemofilie A. De huidige behandeling voor hemofilie patiënten bestaat uit frequente toediening van FVIII preparaten. Aangezien deze therapie kostbaar en voor de patiënt belastend is, wordt veel onderzoek verricht naar alternatieve behandelmethodes waaronder gentherapie. Recent onderzoek heeft aangetoond dat gezamenlijke afgifte van FVIII met zijn chaperone-eiwit von Willebrand factor (VWF) de slagingskans van FVIII gentherapie zou kunnen verhogen. In dit proefschrift is de toepasbaarheid van endotheelcellen als toedieningssysteem voor gezamenlijke afgifte van FVIII met VWF onderzocht.

Een overzicht van de huidige kennis van de biosynthese, opslag en moleculaire interacties van FVIII en VWF wordt gegeven in **hoofdstuk 1.** Hoewel de lever wordt beschouwd als de belangrijkste plaats van FVIII synthese wijst steeds meer onderzoek erop dat endotheelcellen een alternatieve bron voor FVIII vormen. FVIII dat tot over-expressie wordt gebracht in endotheelcellen wordt opgeslagen met VWF in specifieke organellen die Weibel-Palade bodies (WPBs) genoemd worden. Gestimuleerde exocytose van WPBs leidt daardoor mogelijk tot directe secretie van FVIII-VWF complex. Aangezien FVIII in complex met VWF wordt beschermd tegen proteolytische inactivatie en tegen onbedoelde immuunrespons, en bovendien een verlengde circulatietijd heeft, zou afgifte van FVIII-VWF complex uit endotheelcellen een gunstig effect kunnen hebben op de functionaliteit van FVIII.

In **hoofdstuk 2** tonen we aan dat uit bloed geïsoleerde endotheelcellen (BOECs) gestimuleerd kunnen worden tot secretie van VWF uit WPBs met verschillende stimuli. BOECs getransduceerd met een lentivirale vector coderend voor FVIII zijn in staat om langdurig hoge niveaus van FVIII te produceren. Daarnaast wordt een substantieel deel van het gesynthetiseerde FVIII opgeslagen in WPBs en samen met VWF afgegeven na stimulatie van WPB exocytose. Tenslotte hebben we met behulp van confocaal microscopie aangetoond dat FVIII-bevattende WPBs rond zijn, terwijl FVIII-negatieve WPBs hun normale langgerekte vorm behouden.

De rol van VWF als chaperone-eiwit voor het sorteren van FVIII naar WPBs is onderzocht in **hoofdstuk 3**. Hiervoor zijn verschillende FVIII varianten gebruikt die verminderd aan VWF binden. Hoewel de FVIII mutanten een gemiddeld (R2150H, Del2201, P2300S) of ernstig (Y1680F, S2119Y) VWF bindinsdefect hebben, toonden studies in HEK293 cellen en BOECs aan dat alle varianten opgeslagen worden in WPBs. Bovendien bleek de relatieve hoeveelheid FVIII die wordt opgeslagen in WPBs onafhankelijk te zijn van de mutatie in FVIII. Onze data duiden erop dat het sorteren van FVIII naar WPBs niet beïnvloed wordt door een verminderde extracellulaire FVIII-VWF interactie.

De verandering van WPB morfologie die door FVIII expressie veroorzaakt wordt, is in meer detail bestudeerd in **hoofdstuk 4**. We hebben aangetoond dat over-expressie van FVIII de vorming van lange tubulaire VWF structuren verhindert. Deze VWF tubuli zijn verantwoordelijk voor de langgerekte vorm van WPBs. VWF tubuli zijn belangrijk voor de vorming van eiwit-strengen bestaande uit gestrekt VWF (VWF strengen) na exocytose van WPBs. Deze VWF strengen hebben als functie om bloedplaatjes te binden voor de vorming van een trombus. Uit ons onderzoek kwam naar voren dat, ondanks de afwezigheid van lange VWF tubuli in BOECs die FVIII of FVIII Y1680F tot expressie brengen, deze cellen toch in staat zijn

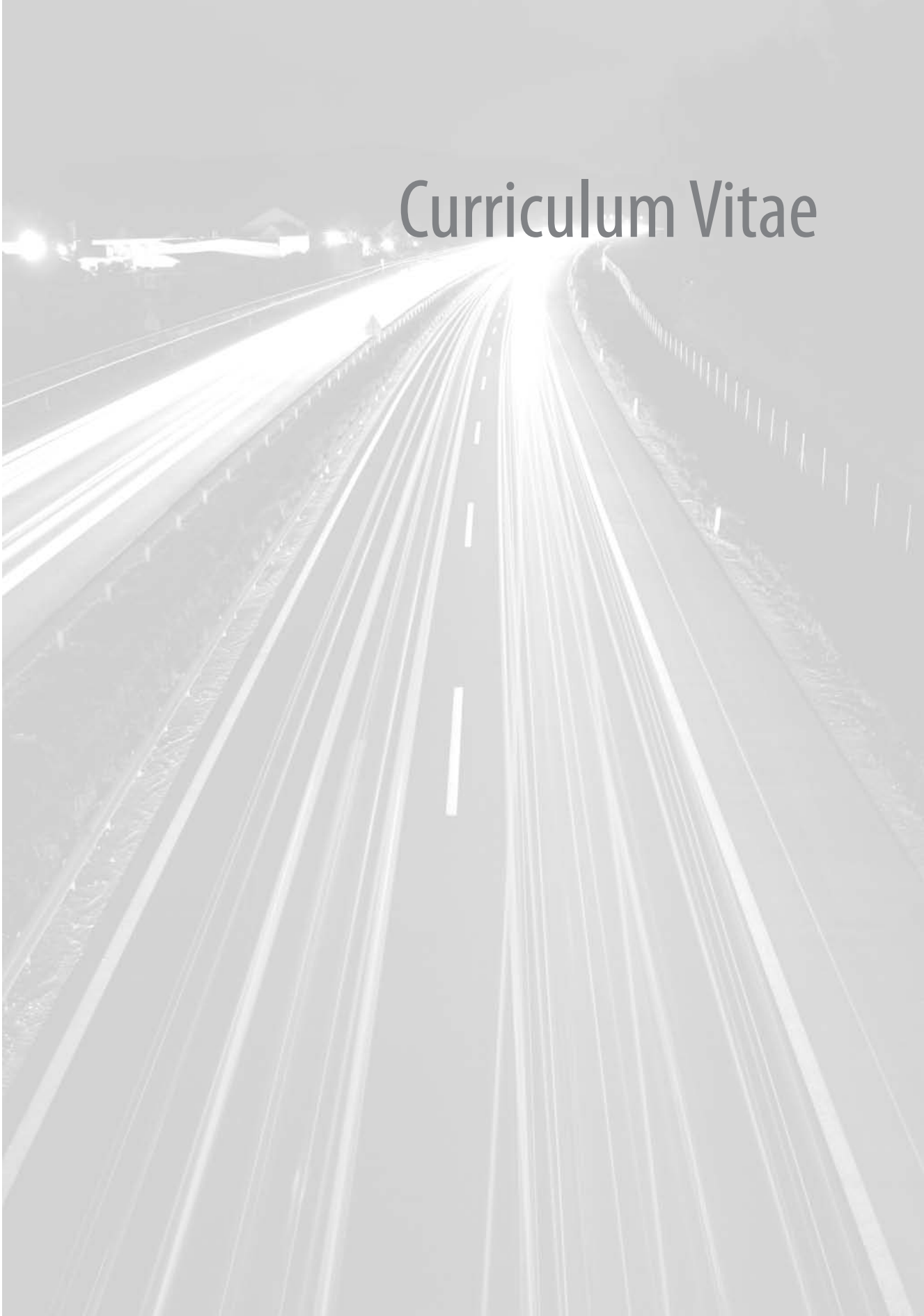
VWF strengen uit te scheiden van gelijke lengte als strengen uit niet-getransduceerde BOECs. Niettemin missen deze VWF strengen grotendeels het vermogen om bloedplaatjes te binden. Deze bevindingen wijzen erop dat een intracellulaire interactie van FVIII met VWF tijdens de vorming van WPBs het binden van bloedplaatjes door VWF strengen voorkomt.

Het effect van FVIII expressie op het opgeslagen VWF suggereert dat FVIII en VWF met elkaar associëren in WPBs. In **hoofdstuk 5** hebben we daarom bestudeerd of FVIII aan VWF gebonden blijft na exocytose uit WPBs. We tonen aan dat FVIII opgeslagen met VWF in WPBs na secretie geassocieerd blijft aan VWF strengen, zonder dat de extracellulaire VWF bindingsplaats Y1680 hiervoor nodig is. Perfusie van VWF strengen uit niet-getransduceerde BOECs met gezuiverd FVIII leidde tot binding van FVIII aan VWF strengen, terwijl FVIII Y1680F in mindere mate associeerde met de VWF strengen. Dit duidt op een verschil in intracellulaire en extracellulaire FVIII-VWF associatie met betrekking tot de bijdrage van aminozuur Y1680 in VWF binding.

De intracellulaire associatie van FVIII met VWF is verder onderzocht in **hoofdstuk 6**. Aangezien factor V (FV) en FVIII structureel homoloog zijn, hebben we hier de mogelijkheid onderzocht dat FV ook naar WPBs sorteert. Hoewel wij wel enig FV in de WPBs aantroffen, bleek de efficiëntie van sortering zeer laag te zijn ten opzichte van die van FVIII. Omdat FV, anders dan FVIII, niet in complex met VWF circuleert, lijkt het aannemelijk dat een interactie met VWF nodig is om voor opslag in WPBs. Verder toonden we aan dat de C domeinen van FVIII essentieel zijn voor het sorteren naar WPBs. Dit vonden wij door deze domeinen te verwijderen of de C domeinen te vervangen voor de corresponderende domeinen van FV. Onze resultaten suggereren dat het sorteren van FVIII naar WPBs wordt gedreven door FVIII-specifieke structurele elementen in beide C domeinen, met de nadruk op het C2 domein.

Tenslotte bespreken we in **hoofdstuk 7** onze bevindingen in het licht van de toepasbaarheid van BOECs als een toedieningsvorm voor FVIII. Daarnaast behandelen we andere in de literatuur beschreven cellulaire afgiftesystemen voor FVIII.

Curriculum Vitae



Eveline Bouwens werd op 9 december 1982 geboren te Geleen. In 2001 behaalde zij het VWO diploma aan 't Alfrink College te Zoetermeer. In 2002 is zij begonnen aan de studie Farmacie aan de Universiteit Utrecht en heeft in 2005 haar bachelor diploma behaald. Vervolgens is zij begonnen aan de master Drug Innovation op de Universiteit Utrecht waar zij in 2008 *cum laude* is afgestudeerd. Tijdens deze studie heeft zij stage gelopen bij de afdeling Clinical Drug Toxicology van de Universiteit Utrecht waar zij onderzoek heeft gedaan naar inductie van cytochroom P450 2C9 door geneesmiddelen ter behandeling van kanker onder leiding van prof. dr. Schellens en prof. dr. Beijnen. Daarnaast heeft zij stage gelopen op de afdeling Plasma Eiwitten van Sanquin Research te Amsterdam onder leiding van prof. dr. Mertens. Hier heeft zij haar master scriptie geschreven over gentherapie voor de behandeling van hemofilie A en onderzoek gedaan naar het gebruik van uit perifeer bloed geïsoleerde endotheelcellen voor de afgifte van factor VIII. In december 2007 is zij begonnen aan haar promotie onderzoek op dezelfde afdeling, waar zij onder leiding van prof. dr. K. Mertens en dr. J. Voorberg werkte aan het in dit proefschrift beschreven onderzoek. Met ingang van juli 2011 is zij werkzaam als post doc aan The Scripps Research Institute in San Diego, USA waar zij de interactie van protein C met de receptor EPCR zal bestuderen.

Dankwoord

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Eveline