Development of Gene Therapy for von Willebrand disease

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Abstract

Von Willebrand disease (VWD) is the most common inherited bleeding disorder in man. It is caused by quantitative or qualitative defects in von Willebrand factor (VWF), a large multimeric glycoprotein that is crucial for normal hemostasis. Treatment of VWD is aimed at restoring normal hemostatic function. Current treatment strategies are based on replacement of the defective VWF protein. Although acute protein replacement therapy or desmopressin-induced VWF release is adequate for the majority of patients, only a short-term effect can be achieved due to the short half-life of VWF (<12 hours). Hence, the search for new and better treatment strategies for VWD is continuing. Gene therapy for VWD offers the potential of a long-term, if not life-long, correction of VWF deficiency, which would dramatically increase the patient’s personal comfort and quality of life. In this review, the current status on the development of gene therapy for VWD is discussed.

Keywords: Von Willebrand factor; von Willebrand disease; gene therapy
INTRODUCTION

In 1926, Finnish physician Erik von Willebrand described a new type of bleeding disorder different from hemophilia among a population in the Åland islands in the Baltic Sea (1). Only decades later, it became clear that clotting factor VIII and von Willebrand factor (VWF) are distinct proteins, with the latter being central to the new disease described by von Willebrand (2). Nowadays, von Willebrand disease (VWD) is well known as the most common inherited bleeding disorder in humans (3,4). The hallmark of VWD is the presence of reduced amounts or abnormal forms of VWF in the circulation. Current therapy only offers a short-term effect, often demanding repeated treatments. Gene therapy for VWD offers the potential of a long-term, if not life-long correction of VWF deficiency, which would dramatically increase the patient’s personal comfort and quality of life.

THE HEMOSTATIC FUNCTION OF VWF

Von Willebrand factor is an intriguingly large, multimeric glycoprotein that plays a crucial role in primary hemostasis. In general, two main functions of VWF are distinguished: recruitment of blood platelets at sites of vascular injury and protection of coagulation factor VIII (FVIII) in the circulation. Biosynthesis of VWF is restricted to endothelial cells and megakaryocytes and involves multiple posttranslational modification steps including removal of the signal peptide and the propeptide, glycosylation, sulphatation and, importantly, multimerization (5). After synthesis in endothelial cells, VWF is secreted constitutively in the circulation and subendothelial space or directed to the Weibel-Palade storage granules, from which it is released upon endothelial stimulation. Following biosynthesis in megakaryocytes, VWF ends up in platelet α-granules, to be released only via a regulated pathway. Once in the bloodstream, VWF circulates with a half-life of 8 to 12 hours.

Multimer size of VWF is pivotal for its function. Larger multimers contain more binding sites for VWF ligands and consequently are more active than smaller multimers. Under normal circumstances, VWF circulates as a mixture of multimers of varying size (up to 20,000 kDa). Ultralarge VWF (>20,000 kDa) is hyperactive and is stored in endothelial and platelet storage granules. After regulated release, these hyperactive, ultralarge VWF multimers guarantee a rapid local hemostatic response upon vascular injury. To prevent accumulation of thrombogenic ultralarge VWF multimers, ADAMTS13 (a disintegrin and metalloprotease with thrombospondin Type 1 repeats 13) cleaves ultralarge VWF into smaller, less reactive molecules (6).

The multi-domain structure of the monomeric VWF building blocks is fundamental to the hemostatic function of multimeric VWF (7). Figure 1A shows the classical domain organization of VWF. Recently, Zhou et al. updated the VWF...
domain structure taking into account new information on the structures of homologous domains in other proteins (8). This resulted in a redesign of VWF domain organization, shown in figure 1B. To maintain normal hemostasis, several domains of VWF are involved in specific interactions. Upon exposure of fibrillar collagen (type I and type III) in the vasculature, VWF rapidly binds via its collagen binding sites (A3 domain and to a lesser extent also A1 domain). Immobilization to collagen and/or high shear forces in blood results in conformational changes that expose the cryptic binding site for platelet glycoprotein (GPIbα in the VWF A1 domain. This permits adhesion of platelets from the circulation to immobilized VWF. Because of the fast on- and off-rates of the GPIb-VWF interaction, platelets are decelerated, allowing the establishment of

Figure 1: Multi-domain structure of VWF. (A) VWF is a multimeric protein composed of dimeric building blocks. The VWF domains, the binding sites of major binding partners and the cleavage site for ADAMTS13 are indicated. (B) Representation of the updated domain structure of VWF(8).
more firm interactions between the platelet collagen receptors (GPVI and integrin \( \alpha_2\beta_1 \)) and the exposed subendothelium. This definitive arrest eventually leads to platelet activation. Upon activation, platelets release soluble platelet agonists such as ADP, ATP and thromboxane A2. In parallel, integrins such as \( \alpha_{2b}\beta_3 \) shift to a high-affinity state. Further platelet recruitment is facilitated by binding of activated platelet \( \alpha_{2b}\beta_3 \) to its primary ligand fibrinogen and to the Arg-Gly-Asp (RGD) sequence found in the C1 domain of VWF. The end result is the formation of a platelet-rich thrombus that is further stabilized by the fibrin network generated via the coagulation cascade. This thrombus seals the site of vascular damage and prevents excessive blood loss.

Another factor that contributes to the hemostatic potential of VWF is its stabilizing effect on FVIII. Indeed, when not bound to VWF, the plasma half-life of FVIII is reduced from 12 hours to 1-2 hours. Plasma FVIII binds tightly to VWF via a non-covalent interaction in the VWF D'D3 domains (reviewed in (9)). Although more binding sites are available on mature VWF, only a ratio of 1 FVIII molecule to 50 VWF monomers is found (10). After cleavage of FVIII by thrombin, FVIII is activated and released from VWF to become available as an effective procoagulant cofactor in the coagulation cascade. Hence the FVIII-VWF complex serves a critical role in mediating primary hemostasis and coagulation.

Obviously, VWF is generally known as an important mediator of hemostasis. However, during recent years, interesting new insights are emerging on the involvement of VWF in several processes beyond hemostasis, such as inflammation, venous thrombosis, stroke, angiogenesis, cell proliferation and apoptosis (reviewed in (11)). It has become clear that, while VWF function is crucial for prevention of bleeding, inhibition of VWF could become an interesting treatment avenue in various thrombo-inflammatory pathologies such as ischemic stroke (12).

VON WILLEBRAND DISEASE

Quantitative or qualitative abnormalities in VWF result in VWD, the most frequent autosomally inherited bleeding disorder. Characteristic bleeding symptoms associated with VWD include mucocutaneous hemorrhages, easy bruising, excessive bleeding from minor wounds, epistaxis, menorrhagia, bleeding from the oral cavity or gastrointestinal tract, and prolonged bleeding especially during or after surgery.

VWD is classified into one of the three main types based on partial (type 1) or complete (type 3) quantitative deficiency in VWF or qualitative defects in VWF (type 2) (13). Type 1 VWD accounts for 60 to 80% of all cases of VWD and is characterized by a mild to severe reduction in plasma VWF levels (and parallel decreases in FVIII levels). The majority of type 1 VWD is associated with mutations that lead to reduced expression or intracellular transport of dimeric pro-VWF,
or faster clearance of the protein from the circulation. Type 1 VWD patients present with a wide spectrum of symptoms, from asymptomatic to severe bleeding episodes, depending on the severity of the VWF and FVIII deficiency. Type 3 VWD is characterized by very low to undetectable levels of VWF and associated low levels of FVIII. It is the most severe form of VWD with a prevalence ranging from 0.5 to 5 cases per million. More than 110 distinct mutations associated with type 3 VWD are scattered over the entire VWF gene and disrupt normal VWF synthesis and secretion.

Type 2 VWD affects 10-30% of the patients and is associated with normal VWF levels but abnormal structure or function of VWF. Based on the underlying defect, type 2 VWD is divided in 4 different subtypes: A, B, M and N. Subtype 2A VWD is the most common qualitative variant, characterized by a decreased platelet-dependent function of VWF that is caused by loss of large and intermediate multimers. Genetic defects include mutations in either the propeptide or cystein knot domain, leading to defective assembly and/or mutations in the A2 domain that increase the susceptibility to proteolysis by ADAMTS13. Subtype 2B VWD, is associated with increased and often spontaneous binding of VWF to platelet GPIb. This gain-of-function phenotype is caused by molecular defects residing within the A1 domain of VWF. High molecular weight multimers are lost from plasma, as spontaneous binding of VWF to platelets facilitates proteolysis by ADAMTS13 and also results in faster clearance of the platelet-VWF complexes. In subtype 2M VWD VWF has decreased function despite (near) normal multimeric distribution. The majority of mutations cause impaired binding of the A1 domain to GPIb but also defective binding to collagen (A3 mutation) has been reported. Finally, subtype 2N VWD is characterized by a decrease in the affinity of VWF for FVIII, caused by mutations in the D’ or D3 FVIII binding domain. Patients with severe reduction in FVIII levels (severe type 1, type 3 and type 2N) may additionally suffer from hemophilia-like joint and muscle bleedings.

CURRENT TREATMENT OF VWD

Current treatment of VWD is based on on-demand replacement of the defective protein to stop or prevent severe bleeding episodes. Two main strategies are used today: administration of desmopressin and infusion of plasma-derived VWF/FVIII concentrates.

Desmopressin (1-desamino-8-D-arginine vasopressin, DDAVP) is a synthetic analogue of vasopressin that induces exocytosis of endothelial Weibel-Palade bodies. DDAVP is not expensive, readily available and its effect is virtually immediate, with a three- to fivefold increase of VWF and FVIII levels within the first hour after administration. DDAVP administration is the treatment of choice in type 1 VWD because functionally intact VWF protein is present in the Weibel-Palade bodies of these patients. However, it is not effective in patients with type 3 VWD or in severe cases of VWD type 1.
and has a poor response in most patients with type 2A en 2M VWD. The use of DDAVP is contraindicated in patients with type 2B VWD due to the risk of thrombocytopenia. Because of the limited half-life of VWF, high VWF levels are maintained for only 6 to 8 hours. Subsequent doses are needed in case of severe bleeding, which may lead to mild side effects (facial flushing, headache, and mild tachycardia) or even refractory responses (tachyphylaxis) (14).

For VWD patients who are unresponsive or intolerant to desmopressin, virally inactivated plasma-derived concentrates are used to restore VWF and FVIII levels. Several plasma products are commercially available with varying composition; some products contain high amounts of FVIII whereas others are almost devoid of FVIII. VWF/FVIII products also differ in their percentage of high molecular weight multimers, which are thought to be critically important in correcting the hemostatic defects. Nevertheless, clinical efficacy of VWF concentrates is generally satisfactory without serious adverse effects. Disadvantages are high cost, short-term effects and risk of contamination with blood-borne pathogens. A promising alternative for these plasma-derived concentrates is recombinant VWF, which has recently entered a phase 1 clinical trial (15). In this trial recombinant VWF was well tolerated in patients with type 3 or severe type 1 VWD and showed similar pharmacokinetics with plasma-derived VWF.

VWD AND QUALITY OF LIFE

Health-related quality of life is seriously impaired in patients with VWD and is strongly associated with the bleeding phenotype (16-20). Indeed, patients with severe bleeding phenotypes (VWD type 3 and some severe cases of types 1 and 2) not only have the looming threat of a serious hemorrhage but are also confronted with more general quality of life challenges that have an impact on educational level, work, social activities and family life (17,21). Surgical interventions, even relatively minor procedures, can be associated with a life-threatening risk of excessive bleeding. Arthropathies that result from frequent hemarthroses limit the patient’s mobility and independence (22,23). Daily treatment of patients suffering from frequent gastrointestinal or other bleeding episodes restricts work and social activities. The daily management of symptoms, the unpredictable and potential life-threatening nature of bleeding episodes, and concerns about the future are furthermore associated with anxiety and depression (17). Especially menorrhagia has been reported to have strong, negative impact on overall life activities and health-related quality of life, leading to early-age hysterectomy in a significant proportion of females with VWD (16,24-27). Taken together, it is clear that long-term treatment strategies that offer sustained correction of VWF deficiency would be particularly beneficial for the VWD community. Documented experience on VWD prophylaxis is limited but available data shows that treatment
with VWF concentrates over long periods of time is efficacious and significantly improves quality of life (28-30).

**GENE THERAPY FOR VWD**

In recent years, the option of gene therapeutic approaches for the treatment of VWD has gained interest (4). The idea behind a gene therapy for VWD is permanent correction of the genetic defect rather than relying on repetitive, on-demand administration of the protein. In theory, genetic correction of the defect that causes VWD offers the potential of a (life-long) cure for the bleeding disorder. Since VWD is a monogenic disease, it is a good candidate for gene therapy. Furthermore, VWF is secreted into the circulation, obviating the need for organ- or tissue-specific targeting of the transgene. Even though important safety and ethical issues need to be addressed in the field of gene therapy before establishing routine clinical gene therapy protocols, the potential of correcting VWF deficiency by gene transfer would have a drastic impact on the patient’s life. It is a tempting idea that a single gene therapeutic treatment could result in a long-term correction of VWD. To obtain successful therapeutic responses in gene therapy, vectors should generate high levels of transgenic protein in the target cells. Both viral and non-viral approaches have been studied for VWD.

**VIRAL GENE THERAPY FOR VWD**

The most common viruses in the field of gene therapy are retroviruses, adenoviruses and adeno-associated viruses (AAV). Viral vectors have high gene delivery efficiency but may elicit toxicity and are costly to produce. In the case of VWF, the large size of the VWF cDNA (8.4 kb) (31) restricts its incorporation in clinically relevant vectors such as adeno-associated (maximum 5kb) and lentiviral vectors (maximum 8kb). Together with the complex posttranslational modification necessary for functional VWF, this size restriction has hindered development of gene therapy for VWD in the era during which gene therapy for hemophilia was being fully explored (32). However, in 2002, the group of R. Hebbel elegantly showed that blood outgrowth endothelial cells (BOECS) could serve as an interesting endothelial vehicle for ex vivo gene therapy (33). Being the natural site of VWF production, endothelial cells form the ideal target cells for ex vivo gene therapy for VWD. After isolation of BOECs from VWD patients, these endothelial progenitor cells could be genetically modified via transduction with viral vectors encoding VWF. Cells expressing VWF could then serve as a source of VWF in the patient after successful transplantation. To test this strategy, we isolated BOECs from dogs suffering from type 3 VWD and transduced these cells in vitro with a lentiviral vector encoding human VWF (34). Despite the large size of the VWF cDNA, we successfully incorporated the complete VWF cDNA into the lentiviral backbone without crippling the vectors. When used to correct the VWF defect in canine VWD type 3 BOECs, high
transduction efficiencies were obtained, resulting in efficient VWF expression (Figure 2). Expression was maintained during passaging of the cells, confirming stable integration of the VWF cDNA into the genome of BOECs. Transgene-expressed VWF was correctly processed, containing the whole range of multimers, comparable to that of endogenous VWF produced by endothelial cells from healthy individuals (34). Thus, BOECs contain the natural machinery to guarantee efficient processing and multimerization of transgene-expressed VWF, which was also stored in Weibel-Palade bodies. This is different when VWF is produced by heterologous COS, HEK or CHO cells (34-36), in which case the amount of high molecular weight multimers is decreased in favor of the amount of low molecular weight multimers. As expected from the high quality of multimerization, transgene-expressed VWF produced by BOECs was functionally active. It efficiently bound to platelet GPIb, collagen and FVIII, the main interactions needed for good hemostatic function of VWF (34). This study was the first proof of concept that transgene delivery of VWF via viral vectors can restore VWF deficiency in VWD type 3 endothelial cells, at least in vitro. Further studies are needed to investigate whether such genetically modified autologous BOECs are capable to deliver enough VWF protein in the circulation in the setting of VWD.

**Figure 2: In vitro phenotypic correction of VWD type 3 canine BOECs.** (A) Multimer analysis of VWF present in the expression medium 14 days after transduction of VWD type 3 canine BOECs with a lentiviral vector encoding human VWF (Lenti-CMV-huVWF). (B) VWF immunostaining in cytoplasm and Weibel-Palade bodies (magnification) of the canine type 3 VWD BOECs transduced with Lenti-CMV-huVWF.
More recently, Wang et al. evaluated two other viral gene therapy strategies to correct VWF deficiency, one based on AAV and the other using lentiviral vectors (37). To circumvent the size limitation of AAV vectors, an elegant segmental pre-mRNA trans-splicing system was used, consisting of two AAV serotype 8 vectors, each delivering one half of the VWF cDNA. Upon transfection or transduction of human embryonic kidney cells with the 5′ and 3′ segmental pre-mRNA trans-splicing constructs or both AAV8−VWF5′ and AAV8−VWF3′ viral vectors respectively, VWF multimers were secreted by the cells, albeit with relatively low amounts of high molecular weight VWF. These data were however not recapitulated in vivo as tail vein injections of AAV8−VWF5′ and AAV8−VWF3′ into Vwf−/− mice, which represent a good model for type 3 VWD (38), did not result in detectable transgene VWF plasma levels (37). In a second strategy, lentiviral vectors expressing murine VWF (mVWF) were used. Transduction of A495 cells with these lentiviral vectors resulted in expression of multimeric mVWF in the conditioned medium. However, when newborn Vwf−/− mice were injected intrahepatically with different doses of lentiviral vectors expressing mVWF, only very low levels of VWF were expressed, which obscured proper evaluation of the VWF multimer profile. Despite the fact that only low molecular weight multimers could be visualized on multimer gels, a reduction in bleeding time in some treated animals was achieved (37).

NON-VIRAL GENE THERAPY FOR VWD

Most gene therapy clinical trials have been conducted with viral vectors, many of them achieving clinical successes (32,39). Nevertheless, given the safety issues and the high cost, non-viral gene therapy strategies are being extensively explored as a safer and cheaper alternative (40). Non-viral gene therapy for VWD is also under investigation. From a gene transfer point of view, the liver is a commonly used target organ for both viral and non-viral strategies. Indeed, most viral vectors like retroviral, lentiviral, AAV and “gutless” adenoviral vectors all target the liver when they are systemically administered to the recipients. However, hepatocytes are not natural sites of VWF synthesis. We and others used the non-viral technique of hydrodynamic gene transfer (41,42) to deliver the VWF expression plasmid into the liver of Vwf−/− mice (43).

Short-term correction of VWD using non-viral vectors

To study the capacity of liver cells to produce VWF, a non-viral vector expressing mVWF under control of the hepatocyte-specific human α1-antitrypsin promoter in combination with a truncated 1.4-kb human factor IX intron (HCRHPi) was constructed (43). Hydrodynamic injection of this expression plasmid into Vwf−/− mice resulted in in vivo transfection of hepatocytes and subsequent secretion of the VWF transgene in the circulation of these VWD mice. Surprisingly high levels
of circulating VWF were found, with peak levels reaching 10 times normal wild-type values three days after gene transfer (Figure 3). Some animals maintained transgene mVWF expression for more than 2.5 months (end of experiments) but the majority of treated mice lost expression within the first month after gene transfer (43). Notably, multimer analysis revealed that hepatocytes were capable of executing the complex synthesis of transgene VWF, producing low, medium and high molecular weight multimers. However, multimerization of transgene VWF in hepatocytes was not as efficient as in endothelial cells as the ratio of higher over lower molecular weight multimers was somewhat decreased in the former. Yet, liver-expressed mVWF effectively bound GPIb and collagen. Transgene-expressed mVWF was also capable of binding FVIII, leading to restoration of FVIII levels in the \( V_{wf}^{-/-} \) mice after mVWF gene transfer. Most importantly, VWF gene transfer into

Figure 3: VWF levels and multimer profile after non-viral VWF gene transfer in \( \text{Vwf}^{-/-} \) mice
(A) Levels of VWF (●) and FVIII (▲) after hydrodynamic VWF gene transfer in \( \text{Vwf}^{-/-} \) mice. Plasma pool of wild-type (WT) mice was used as reference (100%). (B) VWF multimer analysis of transgene-expressed VWF synthesized in the liver three days after gene transfer in 3 different \( \text{Vwf}^{+/-} \) animals (mouse 1, 2 and 3) and VWF present in normal murine plasma pool (NMP).
VWD type 3 mice corrected the bleeding phenotype as measured in a tail clipping bleeding assay 3 days after gene delivery (43,44). The functional quality of liver-expressed mVWF was further demonstrated in a FeCl₃-induced thrombosis model in which efficient VWF-mediated platelet adhesion and aggregation is assessed after vascular injury. In this model, wild-type mice form an occlusive thrombus within approximately 15 minutes whereas VWD type 3 mice are not able to form an occlusive thrombus within the first 45 minutes after injury. Vwf⁻/⁻ mice that received mVWF gene transfer formed stable thrombi similar to wild-type animals, again confirming full hemostatic activity of liver-expressed VWF.

This model of liver-mediated transient expression of transgene-encoded VWF is nowadays elegantly being used to study VWF biology and VWD pathogenesis (44-49).

**Long-term correction of VWD using sleeping beauty transposons**

Non-viral gene transfer typically leads to short-lived transgene expression since no genomic insertion of the transgene into the host genome takes place. However, recent transposon technology combines the benefits of a non-viral gene strategy with the integrating capacities of viral vectors. DNA transposons are discrete fragments of DNA that can change their positions within the genome via a conserved cut-and-paste mechanism. The latest generation Sleeping Beauty (SB) transposon technology has shown great promise in achieving long-term transgene expression in various models (50,51). This system is composed of two vectors that are deliverd *in trans*: a transposon plasmid containing the transgene expression cassette, flanked by inverted repeats (ITR) and an expression plasmid encoding the SB100x transposase which binds to the ITR sequences in order to cut and paste the transgene into the host genome (51). The SB technology is currently also being studied in the setting of VWD (52). Preliminary results show stable integration of the VWF cDNA in the liver of Vwf⁺/- mice after hydrodynamic gene transfer of a mVWF-expressing transposon in combination with the SB100x transposase expression plasmid. Interestingly, sustained (> 1 year) but low expression of transgene mVWF could be achieved using a transposon containing the CAG promoter. However, when using a mVWF transposon with the liver-specific HCRHPi promoter, much higher levels of VWF were produced (approximately 75% of normal values). Although these studies are still ongoing, the first results suggest the feasibility of long-term treatment of VWD by non-viral gene therapy. Based on these data, the European E-Rare TRANSPOSMART consortium has been set up, to further pursue this strategy and to fine-tune “cutting edge” gene therapy tools for high and sustained level of therapeutic VWF protein secretion by the liver (http://www.erare.eu/financed-projects/transposmart).
CONCLUSIONS

Since the original description of the bleeding disorder by Erik von Willebrand in 1926, we have much increased our understanding of the disease and dramatically improved management of VWD. Indeed, treatment in those early days was far from adequate, as exemplified by von Willebrand’s index case, who bled to death during her fourth menses at the age of 13. Current strategies, using DDAVP or plasma concentrates, give satisfactory results, and alternative treatment options can further improve or at least broaden the panel of VWD treatment modalities. Development of gene therapy for VWD seemed to be a daunting task in the past. However, the first proofs of principle using various VWF gene transfer protocols have been established. Once successful in larger animal models, it is an appealing thought that also severe VWD patients would eventually be able to benefit from a long-term, potential lifelong, cure by effective VWF gene transfer protocols. This would dramatically advance the management of this sometimes life-threatening bleeding disorder and radically improve the quality of life of these patients.

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