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Restoration of Plasma von Willebrand Factor Deficiency Is Sufficient to Correct Thrombus Formation After Gene Therapy for Severe von Willebrand Disease


Objective—Gene therapy for severe von Willebrand disease (vWD) seems an interesting treatment alternative with long-term therapeutic potential. We investigated the feasibility of targeting the liver for ectopic expression of physiologically active von Willebrand factor (vWF).

Methods and Results—The capacity of transgene-encoded murine vWF to restore vWF function was studied in a mouse model of severe vWD after liver-specific gene transfer by hydrodynamic injection. By using a hepatocyte-specific α1 antitrypsin promoter, a considerably higher and longer-lasting vWF expression was obtained when compared with a cytomegalovirus promoter, reaching maximum vWF plasma levels that are 10±1 times higher than the wild-type level. Liver-expressed vWF showed the full range of multimers, including the high molecular weight multimers, and restored factor VIII plasma levels, consistent with correction of the bleeding time 3 but not 7 days after gene transfer. Importantly, transgene encoded plasma vWF restored proper platelet adhesion and aggregation in a FeCl3 induced thrombosis model.

Conclusions—High ectopic expression of transgene encoded plasma vWF can be obtained after gene transfer to the liver. Liver-expressed vWF was fully multimerized and able to restore proper platelet plug formation in severe vWD. (Arterioscler Thromb Vasc Biol. 2008;28:1621-1626)

Key Words: gene therapy ■ von Willebrand factor ■ von Willebrand disease ■ liver ■ hemostasis

von Willebrand disease (vWD) is the most common inherited bleeding disorder in man, arising from qualitative (type 2) or quantitative (type 1 and 3) defects in von Willebrand factor (vWF), a multimeric glycoprotein that has 2 major functions in normal hemostasis.1 First, vWF mediates the recruitment of free-flowing blood platelets from the blood stream to the site of vascular injury, serving as a bridge linking the subendothelial structures in the damaged vessel wall and the blood platelet receptors glycoprotein (GP) Iib and GPIIb/IIIa. Secondly, vWF functions as a carrier protein for the antihemophilic factor (FVIII). Loss of function of vWF consequently results in a bleeding phenotype, called vWD, with type 3 being the most severe form characterized by a complete absence of vWF protein.2

vWF is synthesized in endothelial cells and megakaryocytes and is constitutively secreted from the endothelial cells in plasma and subendothelium or is stored in endothelial Weibel-Palade bodies and platelet α-granules for regulated release. Although the exact role of the different vWF compartments remains elusive, acute bleeding problems in human vWD are treated by augmenting plasma vWF levels, either by administration of desmopressin or by infusion of blood products containing concentrated vWF and FVIII.2 However, because of the short half-life of vWF (~12 hours2), only a short-term effect can be achieved, often requiring repeated administrations, which may result in a lowered patient response to DDAVP over time. Moreover, administration of blood products increases the risk of blood-borne contaminations.

Alternative and better treatments for vWD are being investigated, and a gene based approach seems an attractive candidate.3–6 Indeed, instead of replacing the defective protein, it is appealing to treat the underlying cause, ie, the defective gene, thereby creating the potential for a long-term therapeutic effect avoiding frequent treatment procedures, thereby drastically increasing the patient’s personal comfort. Moreover, gene therapy for vWD is favorable because the disease is monogenic and vWF is secreted into the circulation
making organ-specific or tissue-specific targeting redundant. We previously described the construction of a lentiviral vector encoding full-length vWF, providing a potent tool for vWF gene transfer applications. However, in vivo gene transfer via systemic administration of viral vectors predominately results in transduction of liver cells, which would be an ectopic site for vWF production. Interestingly, we and others showed short-term liver-mediated vWF expression in vWF knock-out mice after hydrodynamic gene transfer of vWF expression plasmids containing a cytomegalovirus (CMV) promoter. Yet, expression dropped rapidly to undetectable levels within the first week, probably because of the relatively weak potency of the CMV promoter in liver cells.

For the development of liver-directed vWD gene therapy, aiming at the long-term expression of functional vWF protein, a strong and potent promoter in the targeted liver-cells is warranted. In the present study, we therefore examined vWF expression after liver-directed transfer of a potent liver-specific expression cassette, containing an α1 antitrypsin promoter (AAT). Moreover, to assess the efficacy of liver-mediated vWF expression to fully restore vWF function in severe vWD animals, it is critically important to investigate the consequences of vWF expression on all of the processes in which vWF plays an essential role. One of the main functions of vWF is the initiation and further propagation of thrombus formation at sites of vascular injury. However, it is not known whether liver-directed vWF expression would suffice to restore proper platelet adhesion and aggregation, both needed for normal hemostasis at sites of vascular injury. Moreover, it is unclear what the relative contribution would be of the plasma component of vWF versus platelet or subendothelial vWF in restoring these processes in vWD mice after vWF gene transfer. To address these outstanding issues, we therefore included this important hallmark of vWF functionality in our study by assessing the consequences of liver-directed vWF expression in a ferric chloride induced thrombus model in vWF-deficient (vWF⁻⁻) animals.

**Methods**

For the detailed Methods please see online supplement Methods at http://atvb.ahajournals.org.

In vWF⁻⁻ mice, the technique of hydrodynamic gene transfer was used to assess liver-mediated expression of murine muvWF. Two constructs were used, the first containing a cytomegalovirus (CMV) immediate/early promoter/enhancer and the second a liver-specific human α1-antitrypsin promoter in combination with a truncated 1.4-kb human factor IX intron.

Mice were bled at different time points after gene transfer to assess levels of FVIII (COATests) and transgene-encoded muvWF (muvWF:Ag). The same plasma samples were used for ex vivo functional characterization of liver-expressed muvWF, including analysis of multimer patterns, binding to both collagen (vWF:CB) and glycoprotein GPIbα (vWF:BCo) and FVIII binding capacity. To directly evaluate in vivo functionality of liver-expressed vWF, tail clipping bleeding times were measured and platelet adhesion and aggregation at sites of vascular injury was examined using a ferric chloride-induced thrombosis model.

Data are represented as mean±SEM. For statistical analysis, the Student t test was used. A probability value <0.05 was considered as statistically significant.

**Figure 1.** Levels of vWF (CMV promoter: □ or α1-antitrypsin promoter: ●) and FVIII (□) after hydrodynamic vWF gene transfer in vWF⁻⁻ mice. Plasma pool of wild-type (WT) mice was used as reference (100%).

**Results**

Expression of muvWF

Because of the disrupted vWF gene, vWF⁻⁻ mice present with no detectable amounts of vWF in their circulation. After hydrodynamic transfer of 100 μg of muvWF expressing vector, large amounts of plasma muvWF were detected by ELISA (Figure 1). MuvWF expression after hydrodynamic delivery of the vector comprising the CMV promoter was maximal during the first 2 days after gene transfer (166±30 and 164±39% of wild-type on days 1 and 2 respectively, n=7) and decreased gradually to background values 7 days after gene transfer. Interestingly, a remarkably different expression pattern was observed when the liver-specific AAT/ApoE-HCR promoter/enhancer was used, as both the degree and duration of muvWF expression were significantly increased (Figure 1). Maximum levels were obtained 3 days after gene transfer and reached 10 times the value of wild-type animals (1001±116% of wild-type, n=13). Although expression levels gradually decreased after this peak, muvWF expression was sustained for several weeks. Four weeks after gene transfer, 2 mice had lost muvWF expression, whereas the remaining animals still had a mean muvWF plasma level of 15.4±3.9% (n=4). Two animals maintained transgene muvWF expression for more than 2 months (data not shown, measurements were ended after 2.5 months), vWF⁻⁻ mice injected with saline did not express muvWF, and no vWF could be detected in platelets from vWF⁻⁻ mice or in platelets from vWF⁻⁻ animals that received muvWF cDNA (data not shown). All further experiments were performed on mice receiving muvWF vectors comprising the liver-specific expression cassette.

**Restoration of FVIII Levels**

As a result of the vWF deficiency, vWF⁻⁻ mice have reduced activity levels of FVIII. FVIII levels were determined up to 28 days after muvWF gene transfer and as expected, full restoration of FVIII levels in the treated animals was observed. The pattern of FVIII restoration closely correlated with muvWF antigen levels, reaching a maximum three days after gene transfer (1.73±0.14 times wild-type level, n=6, Figure 1).
supplemental Figure II). These activity values are, however, in agreement with our previous findings that, although vWF expressed in ectopic cells is biologically active, it contains less high molecular weight multimers than when expressed in blood outgrowth endothelial cells, which may be related to the presence of the natural machinery for vWF synthesis in the latter. In agreement with our data, overrepresentation of low molecular weight multimers resulted in relatively low vWF:RCO/vWF:Ag ratios of up to 0.50 (range 0.08 to 0.50) in recombinant human vWF produced by heterologous cells (CHO cells overexpressing furin) despite the presence of the full range of multimers. At present it is unclear why these ratios differ between the 2 time points, and further studies are needed to reveal possible mechanisms, eg, variable proteolysis by ADAMTS13.

Apart from recruiting platelets at sites of vascular injury, a second important role of vWF in hemostasis is binding and protecting circulating FVIII. Liver-expressed vWF is capable of binding FVIII (88.0±6.8% and 87.1±10.1% binding capacity 3 and 7 days after gene transfer, respectively, when compared to vWF in normal murine plasma pool, n=4, please see supplemental Figure III), further confirming the good functional quality of the expressed vWF protein.

**Bleeding Time**

Because of the absence of vWF in the vWF−/− animals, they present with a prolonged bleeding time. To assess whether the transgene muvWF had an effect on the bleeding time, the tail clipping bleeding time assay was performed. vWF−/− animals had a bleeding time around 1.5 to 2 minutes (104±19s, n=10), whereas knock-out mice uniformly bled longer than 10 minutes, after which the experiment was ended (Figure 3). Conversely, the bleeding time of vWF−/− animals 3 days after hydrodynamic delivery of muvWF cDNA was corrected in all but 1 animal (133±47s, n=6). Interestingly, 7 days after gene transfer, 6 of the 7 tested animals did bleed longer than 600 s, suggesting that the correction of the bleeding time was lost after this period.

**Restoration of Thrombus Formation**

One of the main hemostatic functions of vWF is the recruitment of flowing platelets at sites of vascular injury to mediate subsequent platelet adhesion and aggregation, necessary for proper wound healing and the prevention of excessive bleeding. We investigated whether transgene-encoded muvWF was able to correct defective vWF-dependent thrombus build-up, which is one of the hallmarks of severe vWD in this mouse model. For this purpose we used the FeCl3-induced thrombosis model and measured the time from injury to occlusive thrombus formation. In wild-type mice, platelets rapidly
adhered to the damaged vessel wall to form stable aggregates that became occlusive within 20 minutes after FeCl₃ application (17.0±1.6 minutes, n=6). On the contrary, because of defective platelet adhesion and aggregation, vWF⁻/⁻ animals had a pronounced longer TTO (53.8±2.0 minutes, n=7). Interestingly, vWF⁻/⁻ mice that received muvWF cDNA showed a complete restoration of vWF-dependent thrombus formation 3 and 7 days after gene transfer with a TTO of 14.3±0.3 (n=3) and 18.3±2.4 (n=4) minutes respectively (Figure 4). When thrombus formation was observed by intravital fluorescence microscopy, no visible differences could be detected in the process (platelet adhesion, rate of thrombus growth, thrombus size) between wild-type animals and vWF⁻/⁻ animals in which the vWF plasma compartment was restored by gene transfer.

Discussion

The liver remains an attractive target for many gene therapeutic applications, given its ability to efficiently produce and secrete therapeutically relevant proteins into the blood. Long-term liver-directed gene expression has been accomplished after systemic administration of retroviral, lentiviral, adeno-associated viral, and “gutless” adenoviral vectors. In addition, liver-directed gene transfer can be achieved by nonviral hydrodynamic gene delivery of expression plasmids. Localized hydrodynamic gene delivery in a restricted area of the liver is currently being explored in clinical trials.

Treatment of vWD by gene transfer had been hampered in view of the large size of the vWF cDNA (~8.4 kb). Nevertheless, we have recently succeeded in overcoming this bottleneck and inserted full-length vWF in a lentiviral vector, without compromising vector performance. Because vWF is naturally synthesized in endothelial cells and megakaryocytes, not much was known about the in vivo physiological activity of vWF synthesized in hepatocytes, which is crucial for developing potential gene therapy strategies for vWD targeting the liver. Though we have recently shown that ectopic expression of fully processed vWF by the liver was possible after hydrodynamic hepatic gene delivery, it was unclear whether transgenic vWF expressed by hepatocytes would retain its full spectrum of functional properties, in particular its capacity to mediate platelet adhesion and aggregation at sites of vascular injury. Moreover, the short-term expression of transgene vWF observed in previous experiments implies that the development of improved vWF expression cassettes is warranted for efficient gene therapy applications. Indeed, a high and sustained expression of the introduced vWF gene is crucial, imposing a careful selection on the promoter. Increasing transgene expression would decrease the required load of viral vectors, thereby limiting the risks of potential viral side-effects.

We selected a liver-specific expression cassette containing an α1 antitrypsin promoter, a hepatic control locus region, and a truncated human factor IX intron A and compared it with the ubiquitously expressed viral CMV promoter. Whereas we observed typical expression kinetics with the CMV promoter that are in line with our previous observations, a dramatic increase in both the degree and the duration of vWF expression was observed using the liver-specific AAT/ApoE-HCR promoter/enhancer. It has indeed been reported that viral promoters, such as the CMV promoter, are inactivated and confer only low levels of transgene expression in livers of animals, whereas in combination with a hepatic locus control region and a liver-specific promoter, long-term expression can be achieved. Still, 2.5 months after gene transfer, no vWF could be detected in the plasma of recipient mice. The exact mechanisms causing a decline in expression after hydrodynamic gene delivery are still not completely understood and are not the main focus of this article. Several mechanisms have been proposed, including promoter inactivation or loss of vector because vectors do not integrate into the target cell genome. On the other hand, a possible immune response against the secreted transgenic vWF protein in the vWF⁻/⁻ mice cannot be excluded, although transient detection of antibodies against the transgene product reportedly had no influence on the persistence of the transgene circulation.

More important, when considering gene transfer based treatments of vWD, is the question whether transgene vWF expressed by the liver can result in synthesis of a fully
A functional protein that can restore vWF function in vWF−/− mice.

A first important function of vWF is its protective binding of FVIII. Our results show that liver-expressed vWF is indeed able to bind FVIII, which is confirmed by restoration of FVIII levels in vivo after vWF gene transfer. Interestingly, although vWF levels rise to approximately 10 times wild-type values, no such increase in FVIII levels was observed. We assume that such a marked increase of the carrier vWF attributable to the potent vWF expression plasmid used, did exhaust the normal FVIII synthesis, impeding a parallel increase. However, more studies are needed to fully understand this issue. The second main function of vWF in hemostasis is its participation in platelet recruitment at sites of vascular injury by binding to GPIb and collagen. Although these activities can be measured ex vivo in collagen binding assays or ristocetin/botrocetin cofactor assays (please see supplemental Figure II), these ex vivo activity assays are somewhat biased toward lower vWF activity/antigen ratios attributable to overrepresentation of the lower molecular weight vWF multimers. However, it was critically important to independently assess the function of transgene-encoded vWF during thrombus generation in vivo. We therefore examined whether the defective thrombus formation, a typical characteristic of vWF−/− mice that is dependent on proper vWF-dependent platelet adhesion and aggregation, could be restored after gene transfer in the FeCl₃ thrombosis model. This well-established model allowed us to assess the contribution of circulating, transgene-encoded vWF versus platelet or subendothelial vWF in restoring platelet adhesion and aggregation at sites of vascular injury.

Our experiments showed that transgene-encoded vWF is indeed able to restore proper platelet adhesion and aggregation after vascular injury, processes that also occur in normal hemostasis and that are essential to guarantee efficient wound healing and prevention of excessive blood loss. These results corroborate the functional quality of the transgenic vWF protein. Because thrombosis is potentially life-threatening, gene therapy for vWD should obviously not result in a thrombotic event. It is therefore encouraging that no spontaneous thrombotic problems were observed in the treated mice, despite the presence of high levels of plasma vWF, suggesting a broad therapeutic window for plasma vWF, at least in the absence of platelet and subendothelial vWF.

Interestingly, these results also show that plasma vWF by itself is sufficient to restore thrombus formation in vWF−/− mice, which is in contrast with earlier studies in vWD pigs and dogs where thrombus formation was still impaired after reconstitution of the vWF plasma compartment using vWF plasma concentrates. Apart from species differences regarding the respective roles of the different vWF compartments, this discrepancy may possibly be related to quantitative and qualitative differences of transgene encoded liver-expressed vWF versus vWF present in plasma concentrates. Remarkably indeed, in a recent study comparing 12 vWF-containing FVIII products that are commonly used in treatment of vWD, 10 of 12 products revealed a low content of high molecular weight multimers, all 10 having less than 36% of high molecular weight multimers of normal human plasma. In comparison, our liver-expressed transgenic vWF (3 days after gene transfer) contains more than 47% of high molecular weight multimers of normal murine plasma.

Another interesting observation is that restoration of thrombus formation is not completely paralleled by the correction of the tail clipping bleeding time. One possible explanation could be the gradual decrease of high molecular weight multimers on day 7, then suggesting that high molecular weight multimers would play a more dominant role in control of bleeding than in maintaining thrombus formation. On the other hand, these results may point toward the need for platelets, transporting and localizing vWF at sites of vascular injury, to control bleeding or at least to correct the bleeding time, as seemed to be the case in studies in pigs but not in dogs. In vWD patients, however, the need for platelet concentrates to prevent or stop bleeding is very rare.

It is nevertheless important to note that measurement of the bleeding time may not always have a good predictive value for the risk of severe clinical bleeding. Despite no or partial correction of bleeding time after infusion of vWF concentrates in vWD patients, major surgical procedures are carried out successfully and spontaneous bleeding episodes are controlled. Whether, however, the TTO is a stronger indicator for clinical phenotypic correction of vWF deficiency is still unclear.

In conclusion, we show that ultrahigh and prolonged plasma levels of vWF can be reached after vWF gene transfer to the liver by using a liver-specific promoter. In addition, we show for the first time that transgene vWF can be produced by the liver both in a quantity and quality able to restore in vivo platelet adhesion and aggregation, one of its main functions in hemostasis. Whether permanent vWF expression by the liver can be achieved after lentiviral transduction is a subject of ongoing research. Apart from providing proof of concept for an efficient liver-mediated gene therapy for vWD strategy, our results contribute to a better understanding of the role of the vWF plasma compartment in the vWF−/− mice. These insights are needed for the correct interpretation of future experiments using these animals.

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Disclosures
None.

References


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