Published in final edited form as:

Thromb Res. 2012 May ; 129(Suppl 2): S46–S48. doi:10.1016/j.thromres.2012.02.031.

Platelet and Endothelial Expression of Clotting Factors for the Treatment of Hemophilia

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Summary

Targeting of FVIII or FIX to platelet synthesis and storage promotes the normalization of hemostasis in hemophilia A and B mouse models. In both instances, this is accomplished through local release of stored FVIII or FIX, and not plasma clotting factor, at the site of vascular injury. Platelet-targeted FVIII is effective even in the presence of high titer FVIII inhibitory antibodies but only in the presence of physiologic synthesis and localization of VWF. Platelet-targeted FIX is clinically beneficial in FIX KO mice, but is not effective in the presence of FIX inhibitory antibodies. The absence of therapeutic benefit of platelet-targeted FIX or platelet-targeted FVIII without co-expressed VWF emphasizes the unique role of VWF in facilitating the efficacy of platelet-targeted FVIII in the presence of FVIII inhibitors. Platelet-targeting as a mechanism of drug delivery might have clinical therapeutic benefit in other hemostatic disorders, but in hemophilia A, this mechanism of delivery protects FVIII from plasma FVIII inhibitors and promotes FVIII's therapeutic efficacy in the local release of FVIII with VWF in the presence of systemic FVIII inhibitors. This potentially enables a viable therapeutic gene therapy for hemophilia A patients with high titer antibodies. Since a number of laboratories are exploring FVIII or FIX modifications that increase in vivo expression (51–54), these approaches will need to be studied to determine if this increased expression or activity results in added efficacy when expressed in the platelet or endothelial cell.

Gene therapy for hemophilia has most commonly been targeted to expression in the liver or other tissues that results in improvement in plasma levels of Factor VIII (FVIII) or Factor IX (FIX). This has been accomplished through intramuscular (1–4), intrahepatic (3;5;6), intrafibroblast (7), or intravascular (8) administration (vector with the clotting factor under the control of a ubiquitous promoter). Most recently, intravascular adeno-associated virus-8 (AAV8)-mediated FIX gene therapy has been carried out in patients with hemophilia B with levels of FIX that improve the bleeding phenotype (9;10). In all of these approaches, the levels of FVIII or FIX attained in circulating blood plasma was the therapeutic goal.

Since we had done some preliminary experiments with platelet-targeted expression of FVIII that resulted in storage of FVIII together with von Willebrand Factor (VWF) in storage granules, we then explored using this strategy for gene therapy of hemophilia A (or, using Factor IX, for hemophilia B).

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Drs. Montgomery and Shi have no conflicts of interest in this manuscript.

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Co-expression of VWF and FVIII

Therapeutic products for replacement therapy of hemophilia A include plasma derived FVIII products that include VWF (Humate P, Alphanate, and Koate) and some recombinant FVIII products are expressed in the presence of VWF to optimize FVIII synthesis and secretion. Then, the VWF is removed from the FVIII by immunoabsorption so that the final product just contains FVIII.

While it is well known that hemophilia A can be cured by liver transplantation, the specific cell that synthesizes FVIII is not clear (11–16). FVIII synthesis has been demonstrated in sinusoidal endothelial cells (17) and in pulmonary microvascular endothelial cells (18;19), but in situ studies of endothelial cells from various vascular beds do not show co-expression of FVIII and VWF (20;21). FVIII has not been demonstrated in megakaryocytes, and normal bone marrow transplantation does not correct hemophilia (22;23). Since we have been interested in the intermolecular relationships between VWF and FVIII for a number of years (24–27), we explored the co-expression of (B-domain deleted) FVIII with VWF in several model systems – endothelial cells, megakaryocytes, and AtT-20 cells (28–31). In each case there was VWF-dependent storage of FVIII. In the megakaryocytes and AtT-20 cells, there was no release of FVIII or VWF unless an agonist was used to induce release. This suggested that directing FVIII expression to hematopoietic stem cells (HSCs) might be a reasonable approach to providing gene therapy to hemophilia patients by using homologous HSCs transduced with a platelet-specific promoter. We theorized that as in cell culture, FVIII would be synthesized and stored only in megakaryocytes where it would co-localize with VWF. We found that when FVIII was induced to express in megakaryocytes that FVIII was not released into the culture supernate (31–34). It has been known for many years that the co-expression of VWF with FVIII facilitates the efficient transport of FVIII intracellularly and some clinical recombinant FVIII preparations are produced with VWF and then the VWF is removed prior to marketing the recombinant FVIII product (35–38).

Gene Therapy of Hemophilia A in a Murine Model even with FVIII Inhibitory Antibodies

Mice were developed in which the FVIII gene was knocked out by targeted disruption of exon 16 or exon 17 and resulted in a viable model of murine hemophilia A (39;40). Targeting expression of FVIII to megakaryocytes and platelets were carried out by two approaches – one using the GPIIb α -promoter (41;42) and the other the α IIb-promoter (32;33;43). Using the latter approach, we developed transgenic mice in which the human FVIII transgene was under the control of the platelet-specific α IIb promoter (2bF8). These mice were bred with the FVIII KO mice to establish a hemophilic model of murine hemophilia A with FVIII only being localized to platelets and megakaryocytes where it was stored in the platelet α -granule. Correction of the hemophilic phenotype was determined by tail-clip survival (43–45). The hemophilic phenotype was corrected even though no FVIII could be demonstrated in plasma and platelet VWF was only 0.75 mU/10⁸ platelets (corresponds to 1% of circulating FVIII in whole blood) (43). Bone marrow transplantation from these mice into lethally irradiated FVIII KO mice demonstrated that the expression of FVIII was maintained in the bone marrow recipients for their full lifespan and no inhibitory antibodies were produced against FVIII. The phenotypic expression was maintained even with high titer FVIII inhibitory antibodies that were passively transferred using plasma from an FVIII-immunized hemophilia A mouse, transferred by splenocyte-transfer from a highly immunized mouse, or by overcoming tolerance by immunization with human FVIII administered with adjuvant I.P. (43;44). Further studies were undertaken using FVIII KO HSCs that were transduced with a lentiviral construct containing the 2bF8 transgene (45). Similar levels of platelet-FVIII were produced and the phenotypic correction was achieved.

Thus, FVIII expression in platelets achieved phenotypic improvement even in the presence of high-titer FVIII inhibitory antibodies (500–20,000 BU/mL) and the phenotypic correction can be achieved when 10–20% of the platelets contain FVIII or if only 5–10% of the bone marrow is transduced. When studying efficacy in the presence of FVIII inhibitory antibodies, it is critical that the FVIII/VWF complex is formed prior to the infusion of FVIII inhibitory antibodies since a preformed FVIII/VWF complex is released from transduced platelet into plasma containing FVIII inhibitory antibodies (43;44). An alternative approach is to infuse the inhibitory antibodies into hemophilia A mice before FVIII infusion but this antibody prevents formation of the FVIII/VWF complex and underestimates therapeutic efficacy (46).

If lentivirus-2bFVIII (LV-2bF8) gene therapy were to be considered in hemophilia patients with inhibitory antibodies, then the gene therapy would need to be initiated with autologous bone marrow in the presence of inhibitory antibodies. In order to mimic this approach, transgenic 2bF8 mice (donor) and FVIII KO mice (recipient) were immunized with FVIII to achieve high-titer inhibitory antibodies. Bone marrow was transplanted from the immunized 2bF8 transgenic mice into the immunized FVIII KO recipients. The engraftment was normal and tail-clip injury was tolerated similar to the mice transplanted without inhibitory antibodies. Thus the presence of active inhibitory antibodies does not preclude this gene therapy approach. The risk of insertional mutagenesis using a lentiviral delivery is still a concern that must be addressed (47;48).

Efficacy of Platelet-Targeted FVIII is VWF Dependent

While FVIII KO mice do not synthesize FVIII, VWF synthesis is normal. Since FVIII forms a complex with VWF in plasma, we wanted to test the effect of VWF on FVIII localization in gene therapy experiments. The 2bF8 transgene was bred onto a VWF^{-/-}, FVIII^{-/-} double knockout background to determine the effect of VWF on the amount of stored platelet FVIII. In the absence of VWF, FVIII was still stored in platelet α -granules but at a significantly reduced concentration (approximately 50% reduction) (43). Thus VWF is necessary for optimal expression and storage of FVIII in platelets. These mice were then subjected to tail-clip challenge and the efficacy of platelet-targeted FVIII in the presence of inhibitors was eliminated. Thus, the relationship of FVIII and VWF not only prolongs the survival of FVIII, but also improves the hemostatic effectiveness in the presence of FVIII inhibitory antibodies.

Gene Therapy of Hemophilia A by Targeting FVIII to Endothelial cells

The other cell type that expresses endogenous VWF is the vascular endothelium in which synthesized VWF is stored in Weibel-Palade bodies that release the stored proteins following agonist stimulation. In a manner analogous to platelet-specific expression, the cDNA for (B-domain deleted) FVIII was placed under the control of the murine, endothelial-cell-specific, Tie2 promoter (T2F8) (49). Transgenic T2F8 mice were developed and bred onto the FVIII KO background. Heterozygous T2F8 mice had plasma FVIII levels of 56 U/dL and the homozygous T2F8 mice had 127 U/dL that correspond approximately to 50% and 100% of normal mouse FVIII. These mice exhibited the expected correction of the hemophilia A phenotype with these levels of plasma FVIII, but unlike the platelet-FVIII mice, FVIII inhibitors inactivated the plasma FVIII and abrogated the beneficial effect of the expressed FVIII. When the T2F8 transgene was bred onto a VWF^{-/-}, FVIII^{-/-} double KO mouse, no plasma FVIII could be demonstrated unless VWF was infused intravenously. Similar to humans with type 3 von Willebrand Disease (VWD) who have FVIII levels of 3–5 IU/dL, infusion of VWF results in the normalization of plasma FVIII. Thus, targeting FVIII expression to endothelial cells may correct the plasma FVIII level to normal in the

absence of inhibitory antibodies, but would not be expected to be efficacious in the presence of inhibitory antibodies. This lack of efficacy also supports the unique benefit from platelet-FVIII targeted gene therapy.

Gene Therapy of Hemophilia B through Platelet-specific expression of FIX

Since platelet-targeted gene therapy for hemophilia A improved hemostasis in FVIII KO mice, we explored whether platelet-targeted FIX was similarly efficacious in hemophilia B (50). This was explored in transgenic FIX KO mice in which the FIX transgene was driven by the α IIb promoter (2bF9) and a similar construct in a lentiviral vector. In both cases FIX was expressed in megakaryocytes and platelets, and the FIX was stored in platelet α -granules. The platelet-targeted 2bF9 significantly improved hemorrhagic symptoms in a manner analogous to platelet-targeted FVIII, but differed in one important aspect. The platelet-targeted FIX was not efficacious in the presence of FIX inhibitory antibodies. This supports the importance of the FVIII-VWF relationship in therapeutic efficacy in the presence of inhibitory antibodies in hemophilia A.

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