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COMMENTARY

Good things come in small packages for hemophilia

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See also Wilcox DA *et al.* Induction of FVIII expression in human and murie megakaryocytes. This issue, pp. 2477–89.

Factor (F)VIII is an essential component in blood coagulation, deficiency of which causes the serious bleeding disorder hemophilia A. For over 50 years, hemophilia A has been treated by protein replacement therapy using plasma-derived or recombinant (r)FVIII products. However, this treatment regimen has significant limitations that include high cost, limited availability for prophylaxis, potential for virus or prion contamination, and development of an immunogenic response to produce FVIII inhibitory antibodies. Recent developments have improved our understanding of the structure and function of the FVIII molecule and have made feasible hemophilia treatment through novel modalities. At the foremost stage are several gene therapy protocols for hemophilia A that have been initiated in humans [1,2]. However, as the first phase of these clinical studies has drawn to a close, it is evident that newer and improved technologies are required for the eventual long-term goal of a life-long cure for hemophilia. Numerous fundamental unresolved questions remain concerning the optimal choice of vector and host target tissue in order to maximize FVIII plasma level and period of expression, while minimizing toxicity and immunogenecity. Two recent studies Wilcox *et al*. [3] and Yarovoi *et al*. [4] present a novel approach for hemophilia gene therapy by localized delivery of FVIII in alpha granules of platelets. What is particularly novel about this approach is the use of a cell type that produces and stores von Willebrand factor (VWF), the protein that stabilizes FVIII in the plasma.

In the plasma, FVIII circulates in a complex with VWF where the ratio of FVIII to VWF subunits is 1 : 50. VWF mediates platelet adhesion at sites of vascular injury where FVIII is essential for the intrinsic pathway of blood coagulation. In addition, the non-covalent interaction of FVIII with VWF is required to prevent rapid clearance of FVIII from the plasma. VWF also contributes to enhancing the FVIII level in the circulation by promoting the release of FVIII its cellular site of synthesis. Therefore, the functions of FVIII and VWF are intimately intertwined.

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What is the best target tissue for delivery of FVIII genes? A priori, it makes most sense to express FVIII at its natural site of synthesis. Since liver transplantation corrects FVIII levels in hemophilia A patients, it is clear that the liver contributes significantly to FVIII in the plasma. FVIII mRNA was detected in murine sinusoidal endothelial cells and Kupffer cells with low levels in hepatocytes [5]. Interestingly, VWF was not detected in the cells that express FVIII, but rather in the endothelium of larger vessels. At present, it is unclear what contribution non-hepatic tissues provide toward FVIII production. Spleen and lymph node transplant, but not bone marrow transplant, can also correct hemophilia A, supporting that reticuloendothelial cells could also provide a significant natural source of FVIII.

Since VWF promotes FVIII secretion and stability, it seems justified to express these proteins in the same cell. Gene transfer experiments have demonstrated the potential for cells to coexpress VWF with FVIII and to secrete the proteins as a complex [6,7]. Indeed, VWF coexpression significantly increases the secretion of FVIII. In addition, when the two proteins are synthesized in a cell that has a regulated secretory compartment, for example an endothelial cell, the FVIII/VWF complex is stored and can be stimulated for release [8]. Although it is unknown whether a natural cell type exists that coexpresses FVIII with VWF, the Wilcox *et al*. and Yarovoi *et al*. studies demonstrate the potential of FVIII and VWF that are coexpressed to be stored in alpha granules of platelets and to be specifically delivered to sites of injury in a manner competent to promote hemostasis. This demonstrates feasibility for the potential of local delivery of FVIII to damaged blood vessels.

Hematopoietic cells have frequently been used as a recipient for gene transfer experiments because they can be easily isolated, propagated, and reimplanted into recipients, thereby providing an *ex vivo* approach for gene therapy. An *ex vivo* approach provides a more controlled environment to minimize infection of undefined cells that could produce unfavorable reactions. Platelets are derived from megakaryocytes, the only hematopoietic cell type that expresses VWF. VWF expressed in megakaryocytes is stored in alpha granules of the platelets. Activated platelets release their alpha granules at sites of injury, providing a localized delivery of the alpha granule contents. For these combined reasons, megakaryotes are a desireable cell to

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target as a recipient for hemophilia gene therapy. Therefore, although FVIII is not naturally expressed in megakaryoctes or platelets, the attributes of being readily accessible, having endogenous VWF, and the ability for granule release at sites of injury are significant reasons to consider this cell type as a recipient for hemophilia A gene therapy.

Wilcox *et al*. describe that production of FVIII in human megakaryocytes and the potential for this delivery system for hemophilia A treatment. Human and murine CD34+ cells were infected with a retrovirus expressing a B-domain deleted form of human FVIII. After cytokine induction to form multiple lineages, including megakaryocytes, FVIII was expressed in a functional form. The FVIII was colocalized with VWF in alpha granules and both proteins were secreted together in response to platelet agonists. FVlII-transduced human megakaryocytes were transplanted into NOD/SCID mice and after 2–6 weeks, the FVIII retained its association with VWF in alpha granules. In a parallel study by Yarovoi *et al*. mice were engineered to harbor a megakaryocyte-specific promoter and FVIII transgene. These mice had 3–9% plasma levels of FVIII, all present within platelets. The results demonstrate that the intracellular capacity for a platelet to store FVIII with VWF is extremely efficient. In fact, there is no evidence that constitutive protein secretion occurs in platelets. When bred into a hemophilia A background, the whole blood clotting time was partially corrected to 3%. These studies show that FVIII stored in alpha granules can contribute to hemostasis in hemophilia A in a mouse model. Together, the two studies demonstrate feasibility for this approach to target FVIII expression to platelets for local delivery in hemophilia A.

There are several important issues to consider in evaluating the potential for this novel therapeutic approach in humans. Indeed, there are several potential advantages of delivery of FVIII via platelets. First, the total amount of FVIII required would be substantially less because the platelet half-life in the blood is approximately 10 days as opposed to 8 h for FVIII. Second, there may be fewer immunological complications in delivering FVIII in platelets since there would be significantly less FVIII circulating in the plasma that could contribute to a B-cell humoral antibody response. In addition, in individuals with circulating FVIII inhibitory antibodies, the local delivery of FVIII may allow it to function before antibody-dependent inactivation occurs. Finally, the coexpression of VWF may assist FVIII production and localization to the damaged vessel. The results reported in these two papers supports that megakaryocytes and platelets have the ability to express biologically active FVIII. However, the level of FVIII expressed in the retroviral-transduced human megakaryocytes was lower ($3\ \mathrm{mU\ mL^{-1}}$ per 10^5 cells) than that obtained through the transgenic expression technology in the mouse ($30–50\ \mathrm{mU\ mL^{-1}}$ plasma). Therefore, for the approach to be feasible in humans, significantly improved levels of FVIII expression need to be obtained.

In addition, Yarovoi *et al*. raise the possibility that FVIII delivered in this manner may actually promote arterial thrombosis. Where platelet expressed FVIII only partially corrected the whole blood clotting time (3%), more efficacious correction, reaching 100%, was observed in a carotid artery thrombosis assay. This would suggest that ectopic expression of FVIII in platelets may preferentially promote arterial thrombosis, consistent with the high density of platelets in arterial thrombi. It will be particularly interesting to perform studies with FVIII mutants that are defective in vWF binding to determine if vWF-mediated delivery promotes thrombotic potential. The relative contribution of platelet-delivered FVIII for arterial bleeds vs. venous bleeds needs to be established before predicting the safety and efficacy of this approach in humans. There is need to test this approach in large models of hemophilia where similar bleeding complications occur as those in humans.

The studies of Wilcox *et al*. and Yarovoi *et al*. do bring into consideration a novel approach for hemophilia A treatment and have certainly raised new questions. Although it is clear that there are multiple approaches to treat hemophilia A in mice, more stringent tests for safety and toxicity need to be developed before testing in humans. Future studies are required to evaluate the ability for any therapeutic approach to have a long-term therapeutic benefit in humans. Certainly long-term correction in large animal models is one crucial step toward this ultimate goal. The only thing we know for certain is that there is a need for a much more mechanistic understanding of the molecular basis of blood clotting and how clotting in platelet rich thrombi differs from that in platelet-poor thrombi. Until that point in time, it may be prudent to direct hemophilia gene therapy towards expression of FVIII in cells that are known to naturally produce the clotting factor.

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