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Ectopic platelet-delivered factor (F) VIII for the treatment of Hemophilia A: Plasma and platelet FVIII, is it all the same?

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Abstract

Hemophilia A is the most common inherited bleeding diathesis and is due to a deficiency of functional coagulation factor (F) VIII. Most patients have a severe deficiency and require a program of prophylactic plus acute infusions of recombinant FVIII to prevent significant joint and other target organ damage. One of the greatest challenges remaining in the care of these patients is that one fifth to third of the patients develop inhibitors to the infused proteins. While a significant portion of such inhibitors can be either overcome or the inhibitors eliminated, some patients with persistent and significant titers of inhibitors need to rely on second tier therapies that are not as effective at preventing significant bleeding morbidity or mortality. A number of groups have been developing therapeutic strategies for FVIII gene therapy for this disorder. Virtually all of these therapies have in common a rise in the plasma level of FVIII, and interpretation of their efficacy is straightforward related to levels achieved. However, several groups have also shown that FVIII can be ectopically expressed in developing megakaryocytes, where although plasma FVIII levels remain undetectable, this FVIII can be released and be effective at sites of platelet activation. Moreover, it is clear that this platelet (p) FVIII is protected to a degree from inhibitors, making pFVIII a particularly attractive strategy for gene therapy for hemophilia A. Yet at the same time, we have shown that pFVIII has a different availability and distribution in a growing thrombus than plasma FVIII. The clinical implications and challenges of these findings as murine and canine hemophilia A preclinical studies go forward with pFVIII are discussed.

Factor (F) VIII biology: intracellular processing and expression

FVIII is a key cofactor in the generation of a blood clot along with activated FIX. FVIII is translated as a 2,351 amino acid (aa) protein, which includes 3 A-domains, 2 C-domains and a poorly conserved B-domain [1,2]. FVIII is a cofactor in FIX activation [3]. The liver and spleen are the main sites of FVIII production [4]. FVIII is synthesized in the rough endoplasmic reticulum (RER) in association with the chaperone protein BiP and requires cleavage by PACE/furin [5]. Transportation to the Golgi specifically requires LMAN1 (ERGIC-53) and MCFD2 [6,7].FVIII is poorly processed in a number of cell lines [8], often leading to detectable apoptosis [9]. Altering FVIII by partial removal of the B domain, or leaving a 226 amino acid (aa) N-terminal fragment with 6 Asn putative N-glycosylation residues, or a FVIII^{F309S} mutation increases secretion without significantly affecting circulation time or co-factor activity [10,11]. In most cell lines, the majority of hFVIII is cleaved, releasing complexed, two-chained FVIII, the heavier N-terminus chain and lighter C-terminus chain, into the circulation. However, FVIII can also be stored in granules [12]. In endothelial cells, trafficking of FVIII to Weibel-Palade bodies is dependent on its carrier, von Willebrand factor (vWF) [13].For ectopically expressed FVIII in developing megakaryocytes, we have shown that FVIII is not secreted, but rather stored in alpha-granules, largely independent of vWF [14].

Clinical challenges in hemophilia A

FVIII deficiency (hemophilia A) is X-linked, affecting ~1:5,000 live male births [15]. Most patients have a severe form with extremely low endogenous FVIII function (<1%) and spontaneous major bleeds. Recombinant FVIII therapy eliminates many of prior concerns with plasma-derived products, but often at a high cost which can exceed $600,000/year [16].Prophylactic infusions of FVIII lead to fewer chronic joint changes or other major complications [17,18]. Therapeutic strategies using prophylaxis have in fact limited the number of joint and other target organ complications, but a remaining major challenge is the development of FVIII inhibitors in 20-30% of patients.[19] Such patients are often treated with FVIII bypass products [20] or with recombinant FVIIa [21] or by inducing tolerance [22] or immune suppression [23,24]. These therapies are often insufficient. Bleeding complications are common because of the lower efficacy of second tier replacement strategies. Subsequently, these patients have more target-organ damage with associated higher costs [25]. A strategy that would provide FVIII replacement in a manner resistant to inhibitors may then be of particular value in the care of these patients.

Novel approaches for the treatment of the hemophilias

Liver transplantation cures hemophilia A [4], but is associated with significant morbidity and mortality. Another non-vector potential therapy for the hemophilias, ribosomal read-through drugs, may prove useful in the treatment of patients with appropriate codon substitutions [27]. Other non-vector strategies include implantation of FVIII-expressing fibroblast [27] or endothelial cells [28]. So far these approaches have resulted in either low efficacy and/or poor long-term expression in animal models. Recent murine studies using either embryonic stem (ES) cells [29] or induced pluripotent stem (iPS) cells [30] that can undergo endothelial cell differentiation and express FVIII have been used as a proof-of-principle for stem cell therapy for hemophilia A. Finally, in FIX deficient (hemophilia B) mouse models,

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gene-editing using zinc finger nucleases to introduce a corrected gene sequence using adeno-associated virus (AAV) delivery has been successful in ameliorating this bleeding disorder [31].

Hematopoietic lentiviral-based gene therapy

Most gene therapy strategies for hemophilia A involve FVIII-expressing plasmids, retroviruses, lentiviruses, adenovirus and AAV insertions into liver or endothelial cells or hematopoietic cells [32,33]. All strategies that target dividing cells, such as hematopoietic stem cells, involve random genomic insertions and an increased risk of oncogenesis. Studies using retroviral gene therapy for X-linked severe combined immunodeficiency patients have found that many patients treated with this retroviral vector developed leukemia [34]. However, newer studies offer more encouraging news: with increased experience and modified conditioning regimens, improved outcomes have been observed without oncogenetic risks after more than a 4-year follow-up of additional immune deficient patients treated with a different retrovirus vector [35].

At the moment, lentiviral vectors are generating the greatest interest in bone marrow gene therapy. These vectors have been attenuated by deletion of virulence genes, have tissue-specific promoters, have insulator elements to limit genomic read-thru along with self-inactivators (SIN) in the long-terminal repeats [36]. They offer the additional advantage of being able to affect cells that are not rapidly dividing with reasonable efficiency. Several groups have used lentiviral vectors with ubiquitous promoters to drive plasma FVIII expression from hematopoietic tissues [32,37,38]. Some of these groups have examined porcine FVIII (instead of human) because the expression of the porcine FVIII is significantly higher than hFVIII despite sharing 83% sequence homology (10-100-fold higher expression) due to improved secretion [39,40]. In a mouse model of hemophilia A, substitution of parts of the porcine A1 and A3 domains into a chimeric protein produced by genetically modified hematpoietic stem cells resulted in high levels of expression of the chimeric protein in plasma of the animals [41]. This initial study used a murine stem cell virus that would only work in murine cells and so subsequently, the group published a study using a lentiviral vector which was used to transfect stem cells which were then injected into lethally irradiated Hemophilia A mice [42]. In this study, the authors reported mean plasma FVIII expression levels of 0.13 units/ml. These studies utilized a CMV promotor so that all cells expressed the chimeric FVIII protein.

Additional reports have appeared on long-term tissue-specific expression of globins in murine and macaque erythrocytes [43]. European clinical trials have begun to test these strategies in patients with thalassemia major and have shown improvement in transfusion needs. Whether these efforts in more immunologically intact patients with lentiviruses will be free of oncogenic concerns remain to be seen [44].

pFVIII gene therapy for hemophilia A

Our laboratory has for more than 10 years been pursuing the targeted delivery of therapeutics to sites of vascular injury using platelets to modulate clotting, enhance fibrinolysis or inhibit angiogenesis. We have published the development of anti-thrombotic thrombocytes that deliver ectopic urokinase in platelets to sites of injury with no systemic fibrinolysis [45]. We have also demonstrated the use of platelet-specific delivery for the ectopic expression of pFVIII in the treatment of hemophilia A. Initial motivation for these studies was that hemophilia A is relatively common and is often associated with the development of inhibitors. We hypothesized that platelet-stored FVIII might be protected from such inhibitors. Moreover, the structurally and functionally related protein FV [46] was synthesized in megakaryocytes and stored in alpha-granules to be released at sites of injury [47]. Finally the carrier protein vWF for FVIII is normally expressed and stored in alpha-granules [48], and perhaps might enhance the targeting of FVIII to the same granules.

Studies of pFVIII transgenic mice wherein the FVIII expression is driven by the proximal 2.5 kb of the *GpIba* promoter [49] showed that B-domainless human pFVIII (phBFVIII) is specifically stored in alpha-granules and released following platelet activation [50]. The maximal platelet antigen level achieved was equivalent to an ~10% antigenic plasma correction. The gold standard for correction of the murine FVIII^{null} mice was overnight survival after a tail vein snipping as FVIII^{null} mice uniformly die by morning [51]. Our pFVIII transgenic lines/FVIII^{null} mice survived this injury, however, we observed that pFVIII transgenic lines/FVIII^{null} mice with no detectable pFVIII levels also survived. We concluded that this assay is too sensitive, and that this model is actually an exsanguination model, wherein hypovolemic mice shunt blood away from their tails, leading to blood/platelet stasis and an exaggerated efficacy of miniscule amounts of pFVIII [52]. We, therefore pursued other models for testing hemostasis. In a cuticular bleed model, where we avoided exsanguination by limiting the test to 6 hrs, hemostatic improvement in the various pFVIII transgenic lines/FVIII^{null} mice was directly related to measureable pFVIII levels [45]. Using an in situ cremaster laser injury system where we could study the details of platelet and fibrin accumulation in both arterioles and venules, we showed that phBFVIII improved clotting in both beds [52]. However, these studies also showed that the temporospatial availability of phBFVIII differed from infused hBFVIII, resulting in full normalization of the time to onset of fibrin clot formation, but with limited total fibrin/platelet accumulation, likely due to a documented increased embolization compared to infused hBFVIII. Whether this embolization is due to the actual distribution of phBFVIII within the forming thrombus or because of too low levels of expression remains an unanswered question and further studies to increase the available FVIII using strategies to increase expression may help to define the underlying reason for this phenomenon. The biologic relevance of the embolization (in terms of risk of thromboembolic phenomenon) is not clear.

We found that the level of pFVIII in mice that were vWF$^{-/-}$ was 75% of that seen in vWF$^{+/-}$ or vWF$^{+/+}$ [14]. We hypothesize that this decrease is due to FVIII stability in the absence or presence of carrier vWF in the alpha-granule affecting survival over the platelet circulating life. We also showed that the pFVIII in vWF$^{-/-}$ mice was biologically available and active in vivo using a $FeCl_3$ carotid artery injury model [14]. We have shown that this clotting model has a clear dose-response in FVIII^{null} mice infused with hBFVIII [50]. Whether platelet alpha-granule vWF affects pFVIII targeting, stability and efficacy will be of importance in canine studies because these animals lack or are deficient in pvWF [54,55].

Two other groups have published murine studies expressing pFVIII, but used both transgenic and lentiviral approaches [56,57]. Neither group achieved as high pFVIII levels as in our studies, perhaps due to their selected promoter constructs, and both groups relied on the tail exsanguination model to demonstrate efficacy. Of significance, Shi, et al., first demonstrated that pFVIII remains effective in the presence of circulating inhibitors [56,58]. They reported that pFVIII was >10^6-fold more effective than infused hBFVIII. We studied this

issue concurrently using the $FeCl_3$ carotid artery injury model [59] and confirmed improved efficacy of pFVIII in this setting, but showed a benefit of ~10^2-fold compared to infused hBFVIII, and this large degree of difference in efficacy likely reflects on the different hemostasis models used and the efficacy of pFVIII in each model.

Our studies [59] also showed that the presence of anti-FVIII antibodies did not result in an immune-based thrombocytopenia in pFVIII-expressing mice, further supporting the concept that pFVIII in alpha-granules may be sheltered from inhibitory antibodies. Circulating platelets take up immunoglobulins and store them within alpha-granules [60]. We showed that this was true for infused circulating inhibitors in $FVIII^{null}$ mice [59]. Recently, the physical distribution of platelet alpha-granule content has been shown to consist of multiple distinct subpools [61,62] or that the alpha-granules are elongated and have different physical distribution of their protein content within each tubular granule [63]. Moreover, subpools of platelet granules may degranulate differently to various agonists [64]. We interpret the finding that pFVIII is efficacious in the presence of circulating inhibitors to mean that either the inhibitors are stored physically removed from the pFVIII and perhaps differentially released, allowing FVIII activity to be preserved by the pFVIII or that pFVIII bound to vWF is protected from the inhibitors [59] in the circulating platelets.

So far, the maximal level of pFVIII achieved in mice has been ~10% of plasma level. Whether higher levels or specific activity can be more efficacious in $FVIII^{null}$ mice was tested by us both transgenically and by lentiviral pFVIII delivery using a human variant termed IR8 that is resistant to thrombin inactivation [64], but that also binds poorly to vWF; and canine (c) BFVIII, that both has higher specific activity than hBFVIII and is expressed better than hBFVIII in many cell lines [65]. Both variants were more effective than hBFVIII in the cuticular bleeding and $FeCl_3$ carotid artery models [66]. However, in the cremaster injury model, only pcBFVIII was more effective, markedly increasing clot stability. Because inhibitors of FVIII are stored in platelet granules and IR8 is not protected by binding to vWF, we also tested whether pIR8 was effective in the face of inhibitors and found that pIR8 is protected from the inhibitors. Thus the concern that in the dog hemophilia model that the lack of pvWF may limit the ability to study pFVIII biology in the presence of inhibitors is decreased.

Of note, pcBFVIII levels were a third of that seen with hBFVIII expression both in the transgenic mice and in mice whose marrow was reconstituted with lentiviral delivery [66] using cDNAs that in baby hamster kidney (BHK) cells resulted in cells expressing three-fold more cBFVIII than hBFVIII [65]. Given the known limitations of FVIII intracellular processing, resulting in cellular apoptosis [9], we propose that the limitation in level of FVIII that several groups have been able to achieve in developing megakaryocytes, and the decidedly lower levels of cBFVIII that can be achieved in megakaryocytes is the result of intracellular processing of FVIII in megakaryocytes which may be rate-limiting and that the pFVIII is causing apoptosis. If this hypothesis is supported it would imply that attempts to enhance pFVIII levels in a megakaryocyte may be limited and that pFVIII megakaryocytes are at a disadvantage at marrow reconstitution relative to wildtype cells. Studies to further define the potential role of apoptosis in expression levels are underway. It is not clear whether the chimeric porcine/human FVIII which has been used in some transplantation models [41,42] would be better secreted than even the cBFVIII, but studies showing therapeutic plasma FVIII levels in mice are encouraging.

Incipient canine studies

Given the potential advantages of pFVIII in the presence of circulating inhibitors, it is clear that pFVIII may have clinical utility. At the same time, pFVIII has a different distribution both spatially and temporally from plasma FVIII [53], and may be present at low levels in platelet-poor thrombi and especially high in platelet-rich thrombi. Additionally, its interactions with vWF and inhibitors may be quite different from plasma FVIII. It is therefore clear that more studies would be needed before pFVIII can come to clinical fruition as an alternative to other approaches that enhance plasma FVIII levels.

One additional set of concerns comes from the initial report of canine studies [67]. Given the fact that hemophilia A dogs are being treated to enhance pFVIII levels, myelosuppression for bone marrow transplant, leading to thrombocytopenia may be life-threatening. Animals may have a greater bleeding diathesis too if final platelet counts are lower than pre-treatment levels. Milder marrow suppression may limit the percent of platelets expressing pFVIII and this too may further limit efficacy. As mentioned above, the maximal level of pFVIII achievable in a megakaryocyte may be limited so that the only option to increase efficacy is to develop pFVIII of higher specific-activity than hBFVIII, like the cBFVIII discussed above, but which at the same time has as good or better than hBFVIII intracellular processing.

Summary

Platelets offer an opportunity to target therapy to a site of vascular injury. Clearly, ectopically expressed FVIII is stored in alpha-granules and released at a site of injury and is effective to some extent in $FVIII^{null}$ mice. Moreover, this FVIII appears to be resistant to some degree to the presence of circulating inhibitors, and this feature of pFVIII is its most appealing clinical value. On the other hand, pFVIII is not equivalent to plasma FVIII. In some settings it may be less effective and in others more, perhaps associated with thromboembolic complications. The impact of pFVIII on developing megakaryocytes needs to be addressed as pFVIII may limit megakaryocyte maturation and increase apoptosis. Finally a strategy to optimize pFVIII levels and specific activity while avoiding prolonged thrombocytopenia in hemophilia A individuals will be challenging and require additional intermediate animal models compared to therapies that enhance plasma levels.

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