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REVIEW ARTICLE

Gene therapy, bioengineered clotting factors and novel technologies for hemophilia treatment

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Summary. The World Federation of Hemophilia estimates that of the 400 000 individuals worldwide with hemophilia, 300 000 receive either no, or very sporadic, treatment. Thus, considerable innovation will be required to provide cost-effective therapies/cures for all affected individuals. The high cost of prophylactic regimens hampers their widespread use, which further justifies the search for novel cost-effective therapies and ultimately a cure. Five gene transfer phase I clinical trials have been conducted using either direct *in vivo* gene delivery with viral vectors or *ex vivo* plasmid transfections and reimplantation of gene-engineered cells. Although there was evidence of gene transfer and therapeutic effects in some of these trials, stable expression of therapeutic factor VIII or FIX levels has not yet been obtained. Further improvements of the vectors and a better understanding of the immune consequences of gene transfer is warranted, as new trials are being initiated. Bioengineered clotting factors with increased stability and/or activity are being validated further in preclinical studies. Novel clotting factor formulations based on PEGylated liposomes with prolonged activities are being tested in the clinic, and are yielding encouraging results.

Keywords: coagulation, factor VIII, factor IX, gene therapy, hemophilia, vector.

Introduction

Incremental advances in our understanding of the biology of coagulation and in preclinical and clinical research suggest several strategies that may facilitate both the prevention and improved management of factor VIII and IX (FIX) deficiencies. Moreover, the research is not occurring in a vacuum: advances in gene and protein engineering in other diseases, coupled with thrombosis research, offer insights into more effective intervention in the clotting factor disorders. Moreover, the technologies may be synergistic: gene delivery of bioengineered clotting factors may overcome some of the inherent delivery and dosing issues that have confronted gene delivery of native molecules. This review summarizes some of the notable recent advances in the field. The common objective of these studies is to develop novel therapies for hemophilia that would either obviate the need for repeated clotting factor infusions by gene therapy or potentially reduce the frequency of treatment using potentially longer-acting or more active biologics, such as novel bioengineered clotting factors, mimetic antibodies or small oral drugs that suppress nonsense mutations.

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Gene therapy: advances in vectorology

Lentiviral and retroviral vectors: benefits and limitations

Most of the current strategies for hemophilia gene therapy rely on viral vectors derived from retro/lentivirus or adeno-associated virus (AAV), which have significantly reduced inflammatory properties in comparison with adenoviral vectors [1]. Lentiviral and retroviral vectors integrate stably into the target cell genome, allowing for long-term transgene expression, and thus are well suited for gene transfer into dividing stem/progenitor cells.

Using either transgenics or lentiviral transduction of hematopoietic stem/progenitor cells (HSCs), FVIII expression can be obtained in platelets [2]. As FVIII is stabilized by platelet von Willebrand factor, only small amounts of FVIII were needed to achieve phenotypic correction. Remarkably, FVIII functioned even in the presence of high-titer inhibitory antibodies, and the bleeding diathesis in FVIII knockout mice could be corrected in the absence of detectable circulating FVIII levels. This may represent a unique advantage over protein replacement therapy. Alternatively, lentiviral vectors can be used to express therapeutic FIX levels in the erythroid lineage [3]. Additional safety is conferred as the red cells become terminally differentiated and enucleated. However, ectopic expression of FIX in the erythroid lineage did not yield

fully active proteins, due to limiting post-translational modifications. One possible limitation of using HSC-based approaches for hemophilia gene therapy is that myeloablative conditioning is required to facilitate engraftment in the bone marrow niches [4]. To obviate the need for myeloablative conditioning, an alternative strategy was developed based on the use of muscle stem-cell-derived retrievable implants composed of non-dividing muscle fibers. This technology could be adapted for FIX delivery as a potential 'reversible' gene therapy for hemophilia B [5].

As retroviral vectors only transduce dividing cells, their use for *in vivo* gene therapy is limited to neonatal or juvenile recipients. Neonatal retroviral FVIII gene transfer yielded stable therapeutic FVIII levels in hemophilia A dogs [6], consistent with earlier successful studies in neonatal FVIII-deficient mice [7]. In contrast, lentiviral vectors could transduce non-dividing adult hepatocytes. Intravenous injection of lentiviral–FVIII vectors derived from feline immune deficiency virus resulted in therapeutic FVIII levels in FVIII-deficient mice [8]. Ectopic FIX expression in antigen-presenting cells (APCs) following lentiviral transduction increases the risk of developing inhibitory antibodies (to FIX) and cellular immune responses that eliminate the gene-engineered cells [9]. Even the use of a hepatocyte-specific promoter does not guarantee long-term expression, possibly due to leaky transgene expression in APCs. However, this could be overcome using micro-RNA-mediated gene silencing in APCs, with consequently prolonged transgene expression [10]. Although gene therapy trials for human immunodeficiency virus and b-thalassemia have been initiated using lentiviral vectors, their potential use for hemophilia gene therapy requires confirmation of their safety and efficacy in large animal models.

Risks of insertional oncogenesis

The development of T-cell leukemia in three boys treated by *ex vivo* retroviral gene transfer for X-linked severe combined immunodeficiency (SCID) [11], along with the emergence of tumors following fetal gene transfer with lentiviral vectors [12], raised concerns about the risk of insertional oncogenesis. Insertion of the therapeutic transgene adjacent to the LMO2 transcriptional coactivator locus in the leukemic cells was thought to play an important pathogenetic role, linking gene transfer to the development of the malignancy, along with a selective advantage for newly expressing interleukin-2 $R\gamma_c$-positive T cells. Hence, the risk of insertional oncogenesis in the context of gene therapy for hemophilia is significantly lower, as there is no selective advantage for the gene-corrected cells, and the gene product itself does not influence cellular proliferation.

Benefits of site-specific genomic integration

To obviate concerns associated with random integration, targeted integration may be preferred. This could be accomplished using a two-plasmid system, one plasmid delivering the therapeutic gene and one delivering a phage integrase that catalyzes site-specific integration of the therapeutic gene into specific integration hot spots in the mammalian genome [13,14]. Although studies in transgenic animals showed no developmental or other abnormalities, the safety of this system would need to be carefully addressed, particularly as integrations were accompanied by deletions or chromosomal rearrangements. Moreover, although this phage technology has been explored for *ex vivo* gene delivery, direct *in vivo* administration of the plasmids will require the development of a clinically acceptable, efficient non-viral gene delivery approach.

Adeno-associated viral vectors: new insights

Adeno-associated virus remains one of the favorites for long-term cures of genetic diseases, by virtue of its potential for long-term gene expression and reduced inflammatory properties. Moreover, the majority of the AAV genomes do not integrate, which minimizes the risk of insertional oncogenesis. There are currently several AAV trials ongoing worldwide for different genetic and acquired diseases, and two gene therapy trials for hemophilia B have been completed [15,16]. In the muscle trial, FIX expression levels were subtherapeutic but stable, whereas in the liver trial, expression levels were in the therapeutic range but transient (see below).

Multiyear expression of a B-domain-deleted FVIII (BDD-FVIII) has recently been described in hemophilia A dogs to circulating levels of 2.5–5% of normal with AAV2, AAV6 and AAV8 vectors [17]. The use of alternative AAV serotypes has generally been proposed as a means to achieve higher clotting factor levels in patients, based on their superior hepatic transduction efficiencies in mice [18,19]. Indeed, the highest clotting factor levels and hepatic gene transfer efficiencies could be obtained using AAV8 and AAV9, which were more efficient and exhibited reduced proinflammatory properties in comparison to lentiviral vectors [20]. However, no transduction advantage of using AAV8 over AAV2 or AAV5 vectors was apparent in non-human primates or dogs [17,21–23]. Thus, some caution is warranted in translating results from rodent species to larger animals, which justifies some preclinical development in non-human primates.

Intravenous injection of a self-complementary (sc) AAV8-FIX, encoding a codon-optimized FIX in macaques, resulted in therapeutic FIX levels (20% of normal) [22]. This 'sc' cassette is more efficient because it obviates the need for second-strand synthesis or reannealing of positive and negative strands delivered in AAV. This scAAV vector appeared to show a relatively modest improvement (threefold) in mice compared to the AAV vector design that had been used previously in a clinical trial [15], when packaged into AAV8 particles [22]. This scAAV8 vector will be used in a planned phase I clinical trial in severe hemophilia B patients.

Immune consequences of gene transfer

Therapeutic FIX levels could be obtained following liver-directed gene therapy with AAV2 vectors in patients [15].

However, FIX expression eventually declined, and this was accompanied by transiently elevated transaminases. This was probably due to the elimination of FIX-expressing hepatocytes by an AAV2-specific cytotoxic $CD8^+$ T-cell (CTL) response. This hypothesis will be tested in a new trial, repeating the AAV2-FIX liver delivery but in conjunction with transient immunosuppression to prevent CTL generation. In addition, as the transaminitis was vector dose-dependent, the use of lower vector doses may minimize these side-effects and therefore warrants the development of more potent FIX expression cassettes.

AAV2 capsid peptides were identified as being capable of tight binding to the subject's HLA-B haplotype. However, as these epitopes will vary between different patients, due to the inherent polymorphic nature of HLA class I between individuals, it is unlikely that one could engineer around these binding sites [15]. Moreover, as these sequences are highly conserved in all primate AAVs, serotype switching may not suffice to overcome this immune rejection [15]. Although the more rapid turnover of AAV8 capsids vs. AAV2 may perhaps diminish this risk [19], this may not necessarily be the case in human subjects, as AAV8 capsids persisted for at least several weeks in non-human primates [23]. AAV capsid processing and presentation in human hepatocytes and APCs would need to be better understood. Nevertheless, one potential advantage of using AAV8 capsids over AAV2 capsids is their reduced uptake by APCs, which may perhaps reduce the risk of CTL induction [24]. Whether AAV8 will eventually prove beneficial over AAV2 awaits confirmation in clinical trials.

A second significant problem when one contemplates treating patients with hemophilia [15] is the presence of pre-existing antibodies to AAV, which can abrogate AAV transduction, even at low titer [25]. As these antibodies variably cross-react with AAVs acquired during natural infection, accurate vector dosing in patients will be problematic, unless compartmentalized delivery is used, such as in isolated limb perfusion, which yielded robust FIX expression (4–14% FIX) in hemophilic dogs [26]. Interestingly, AAV2 variants obtained by either exon-shuffling or by introducing specific mutations into the capsid exhibit reduced cross-reactivity with AAV2-specific antibodies [27,28]. The use of AAV variants isolated from non-human or non-primate species with reduced cross-reactivity to AAV2 may obviate pre-existing immunity, provided that transduction of human cells is not compromised, which is not necessarily the case [29].

It is not known whether gene therapy would increase or decrease the likelihood of inhibitor formation in hemophilic patients compared to protein replacement therapy. One possible concern is that the use of viral vector preparations may provide immunologic 'danger signals' that may facilitate inhibitor formation [30,31]. It is encouraging, however, that hepatic gene delivery of FIX using AAV vectors resulted in FIX-specific immune tolerance, possibly involving $CD4^+CD25^+$ regulatory T cells (T_{reg}), and actually prevented anti-FIX antibody and CTL formation [32]. Protocols for tolerance induction to the transgene product that selectively expand T_{reg} may therefore hold the key to sustained therapeutic expression. However, these results are at odds with the loss of transgene expression due to the emergence of a memory CTL response against the AAV capsid in the AAV2-based liver trial. Therefore, one has to note that the activity of T_{reg} may be less effective in the context of memory T cells to viral antigens that were originally generated by natural infection.

Bioengineered clotting factors to increase protein expression and bioactivity

Both recombinant protein replacement therapy and gene therapy could be facilitated by either the development of factors that demonstrate improved biosynthesis or through the generation of coagulation proteins with enhanced biological properties. FVIII variants have been generated that either stabilize the molecule, increase the specific activity, or enhance production [33]. BDD–FVIII yields higher mRNA levels, and targeted point mutations within the A1 domain reduce interactions with the endoplasmic reticulum (ER) chaperone immunoglobulin-binding protein. In order to increase ER-to-Golgi transport, several asparagine-linked oligosaccharides within a short B-domain spacer have been engineered within BDD–FVIII. A bioengineered FVIII incorporating a combination of these elements was secreted 15- to 25-fold more efficiently than full-length FVIII, both *in vitro* and *in vivo* [34]. In addition, FVIIIa could be stabilized via the incorporation of a disulfide bond that prevents the spontaneous dissociation of the A2 domain from the activated FVIII heterotrimer following thrombin activation [35]. This disulfide bond-stabilized FVIII showed prolonged FVIIIa activity and improved potency in whole blood clotting assays [36]. Gain-of-function FVIII mutants have also been created by engineering out calcium-binding sites in the FVIII heavy chain, resulting in 2-fold increased activity [37]. Although these different FVIII mutants may confer advantages over the native molecule when delivered either as proteins or as genes, their efficacy and safety would still need to be confirmed in hemophilic models.

The specific activity of FIX could also be increased by protein engineering. In particular, replacing the first epidermal growth factor (EGF)-like domain of FIX with that of factor VII (designated FIX–EGF_{FVII}) resulted in a bioengineered FIX molecule with improved bioactivity in hemophilia B dogs [38]. Engineering out the collagen IV-binding sites of FIX improved the bioavailability of FIX, resulting in higher circulating FIX levels when it was delivered via gene therapy [39]. Alternatively, the half-life of FIX could be improved further by generating a fusion protein comprising FIX and the immunoglobulin (Ig) constant (Fc) region in a unique monomeric configuration. This FIX–Ig fusion protein exhibited improved pharmacokinetic properties and clotting activities in hemophilia B mice and dogs (R. Peters, personal communication).

Whether the use of bioengineered clotting factors alters the risk of inhibitors remains to be addressed. Some gain-of-function mutations are buried, or conservative, and thus would not be expected to result in new epitopes recognizable by B

cells. In fact, some mutations may decrease the risk of inhibitor formation, permitting a longer-term strategy to develop more active, less immunogenic, molecules [40].

Novel technologies that can affect the coagulation cascade

Mimetic antibodies as substitutes for FVIII cofactor activity

One of the cofactor functions of FVIIIa to promote activation of FX is to support the appropriate interaction between FIXa and FX. Bispecific antibodies have been developed that mimic FVIII ('mimetic antibodies') and facilitate such an interaction (H. Saito, personal communication). Some of these bispecific antibodies accelerated FX activation in the absence of FVIIIa *in vitro* and shortened the modified activated partial thromboplastin time (aPTT) in FVIII-deficient plasma. These results demonstrate that the cofactor function of FVIII can be mimicked by bispecific antibodies to FIX and FX, and suggest that these mimetic antibodies could potentially act as long-acting therapeutics for hemophilia A, even in patients with inhibitors.

Translational repair of mutant transcripts

Small molecules have been developed (PTC124) that can read through premature 'stop' codons so that the full protein will be produced (L. Miller, personal communication). Approximately 10% of hemophilia A and B mutations are nonsense changes. The work with PTC124 was based upon early observations with aminoglycoside antibiotics, which enabled the testing of PTC124 in phase II clinical trials for Duchenne muscular dystrophy and cystic fibrosis. A pilot study of short-term intravenous aminoglycoside treatment in severe hemophiliacs with nonsense mutations has also shown some promise [41], as increased FVIII levels and decreased aPTTs were noted in some patients. Preliminary results with PTC124 indicate that this oral drug is better tolerated than antibiotics such as gentamicin, and could lead to clinical improvements. Thus, PTC124 may also be useful in a minority of patients with hemophilia with these types of mutations.

PEGylated FVIII to extend the circulating half-life

When mixed with PEGylated liposomes (PEGLip), FVIII binds non-covalently but with high affinity to the external liposome surface. The biological half-life of PEGLip–FVIII is prolonged in mice, resulting in extended hemostatic efficacy of FVIII in hemophilic mice [42]. A recent clinical study showed that, although the plasma pharmacokinetics were identical, the mean number of days without bleeds was significantly prolonged with the PEGLip–FVIII vs. non-PEGylated FVIII [43]. This suggests that PEGLip–FVIII may reduce the frequency of treatment during prophylaxis by extending the period between bleeding episodes.

Conclusions

The rapid pace of this emerging multidisciplinary field continues to fuel the debate on the clinical and ethical issues of developing new therapeutics for hemophilia [44–46]. Despite legitimate differences of opinion, there exists significant consensus on moving both gene- and protein-based research forward using multiple technologies, to develop improved therapeutics, which will hopefully bring a cure for bleeding disorders one step closer to reality. Although the development of a cure by gene therapy may be the ideal solution, there are still some hurdles ahead; however, these are not insurmountable. Moreover, if reduced clotting factor usage can safely be accomplished by using novel bioengineered or PEGylated clotting factors, mimetic antibodies, or even small drugs, this will represent a significant advance over current treatment. It is important to recognize the marked disparities in treatment worldwide, and the need for the development of cost-effective solutions, based upon emerging technologies, to provide for the majority of individuals with hemophilia who have either no, or limited, access to treatment.

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Disclosure of Conflict of Interests

G. F. Pierce is an employee of Bayer Health Care, the other authors state that they have no conflict of interest.

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