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Gene Therapists Determined to Stop the Bleeding!

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As this special issue of the *Journal of Genetic Syndromes and Gene Therapy* illustrates, gene therapists are closer than ever to a cure for the X-linked bleeding disorder hemophilia. Hemophilia A and B are caused by deficiency of functional coagulation of factor VIII (FVIII) or factor IX (FIX), respectively. Patients with severe hemophilia typically require frequent intravenous infusions of recombinant or plasma-derived clotting factor protein [1]. This type of therapy is not efficient enough to prevent bleeding complications and tissue damage unless given in prophylactic manner, which requires even more frequent infusions. A major complication of treatment is formation of inhibitory antibodies, which occurs in a subset of patients and further complicates treatment. Another major challenge for recombinant protein therapy is the high cost, which can be more than $300,000/year in factor products. Thus, hemophilia has been viewed as an ideal candidate for gene therapy, which holds the promise of rendering the patient's own cells into a factory for continued production of functional coagulation factor.

Recent Clinical Success with AAV Gene Transfer

While several early gene therapy strategies appeared promising in laboratory animals, these treatments largely failed to provide a sustained clinical effect [2]. However, the team of scientists who pioneered clinical gene transfer for hemophilia using adeno-associated virus (AAV) vectors persevered through these setbacks to change the outlook [3-5]. An international team of investigators (including scientists at the University College London, St. Jude's Children's Hospital, and the original groups at The Children's Hospital of Philadelphia and Stanford University) has now reported a groundbreaking study, in which liver-directed AAV gene transfer resulted in sustained therapeutic FIX expression in several patients with hemophilia B [6]. This result provides a great stimulus to propel the exciting field of gene therapy for hemophilia forward.

There are several advantages of using recombinant AAV, which makes it a desirable therapeutic vector for a number of diseases [7,8]. AAV vectors are derived from a non-pathogenic virus and have a good safety profile. They are efficient in transferring genes *in vivo* to a number of cell types, which has resulted in many years of transgene expression in several large animal studies and in humans [9]. These characteristics of AAV make it a very beneficial vector for hemophilia A and B gene therapy. The aforementioned landmark study by Nathwani et al. reported the significant finding that a single intravenous administration of an AAV vector could direct expression of therapeutic FIX levels for at least 22 months, thereby eliminating the need for frequent factor infusions [6]. In this Phase I/II dose-escalation study, a total of six patients were treated with serotype 8 vector, which has strong *in vivo* tropism for hepatocytes [10]. While all patients received a therapeutic benefit, FIX levels were vector dose dependent [6]. Four of the six patients stopped conventional treatment altogether, and the other two have extended the time period between prophylactic injections. From a financial perspective, treatment of one hemophilic patient costs can add up to $20 million for a lifetime of treatment. Utilizing gene therapy, this cost could be greatly reduced to roughly $30,000 per year since the use of factor concentrate would be highly reduced or removed.

Future challenges for the protocol include pre-existing immunity to AAV (because of natural infection with the wild-type virus) in the form of neutralizing antibodies (that are present in some patients and block gene transfer) and in the form of memory $CD8^+$ T cells to the viral capsid. Upon reactivation, these may destroy transduced hepatocytes. Multiple potential solutions are being investigated, ranging from vector engineering to transient immune suppression. For example, elimination of surface exposed tyrosine residues can improve effective of AAV vectors in gene transfer, thereby allowing for a reduction in vector dose and thus in lower capsid antigen doses [8].

Remaining Challenges for AAV-Based Gene Therapy for Hemophilia

Future use of the protocol in pediatric patients with hemophilia is complicated by the predominant episomal nature of the AAV genome, which on the one hand reduces the risk for insertional mutagenesis while on the other hand vector genomes are lost over time in a growing liver. However, a recent paper showed that genome editing with zinc finger nucleases offers the exciting possibility of inserting the AAV vector genome into specific places of the host genome, thereby creating a stable form [11]. Thus far, clinical trials with AAV vectors have focused on hemophilia B, i.e. FIX deficiency. FVIII is a larger protein and therefore more difficult to package in vectors and express at therapeutic levels. This makes clinical success in hemophilia A more challenging. To remedy this, bioengineering FVIII has been a priority [12]. Bioengineering approaches include manipulating DNA or RNA via codon optimization and manipulation at the protein level such as altering the FVIII amino acid sequence for improved expression and secretion or to reduce the size of the protein. Combined with other vector engineering strategies, these efforts can also help the use of AAV vectors, which have limited gene-packaging capacity. However, one must proceed with caution since the risk of immune responses against these new molecules needs to be evaluated.

Alternative Viral Vectors for Hemophilia Gene Therapy

Another important aspect to assure future success of gene therapy for hemophilia is that a number of promising alternative approaches are in the pipeline, utilizing a variety of vectors and target cells for transfer of the therapeutic clotting factor genes. For example, lentiviral vectors (LV) may circumvent pre-immunity to the vector and can transduce both dividing and non-diving cells [13]. Also, LV have a larger packaging capacity than AAV vectors and can therefore accommodate

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larger gene constructs. *In vivo* gene transfer with these vectors is still hampered by inefficient vector production, which is not an issue for *ex vivo* gene transfer, e.g. to stem cells. The main safety issue related to LVs is their potential for insertional mutagenesis. Although new generation LV technologies have safety features that can reduce this risk, such issues must be resolved before advancing to clinical trials. As with other treatments, there is the potential risk for the development of inhibitors to the clotting factors using LV, which can be minimized by preventing the ectopic expression in antigen-presenting cells.

Helper-dependent adenoviral vectors (HDAd) are another type of viral vector that is being developed for hemophilia gene therapy [14]. These vectors do not contain viral coding sequences, which gives them considerable cloning capacity due to the large genome size. These vectors also have the capability to transduce many cell types and mediate long-term expression without chronic toxicity. However, a remaining concern is higher innate immunity to this vector, which could cause toxicities early after administration. Improved delivery technologies may circumvent this problem.

Non-Viral Approaches

To avoid complications of *in vivo* gene transfer, stem cells can be transduced *ex vivo* followed by implantation of the gene-modified cells. Future protocols may utilize embryonic stem cells or induced pluripotent stem cells. Currently, hematopoietic stem cells (HSCs) have been most advanced toward gene therapy for hemophilia [15]. HSC can reconstitute all hematopoietic lineages, which allows for expression from multiple cell types or, alternatively, transcriptional targeting of transgene expression to a specific cell type of the blood. A major emphasis of these studies has been optimization of FVIII transgene expression in hematopoietic cells and evaluation of safety concerns that arise from the potential for insertional mutagenesis. LV will likely be a better choice than the originally used murine retroviral vectors. Interestingly, HSC gene transfer has also been described to induce immune tolerance to FVIII. Targeting gene expression to platelets represents an unusual and innovative strategy that may even deliver therapeutic factor protected from antibodies, making it a suitable strategy for treatment of patients with inhibitors [16]. Usefulness of platelet specific delivery for the expression of FVIII in murine hemophilia A has been demonstrated. However, platelet-derived FVIII may increase apoptosis and limit megakaryote maturation, necessitating further optimization in the appropriate animal models.

While viral vectors take advantage of the natural ability of viruses to efficiently infect cells, alternative non-viral gene delivery methods could potentially avoid some of the complications that arise from use of viral particles and genomes such as immune responses (including pre-existing immunity) and insertional mutagenesis. Non-viral therapeutics may also be simpler and cheaper and have a longer shelf life [17]. With no capsid proteins to cause an immune response in the host and therefore no subsequent memory response, these non-viral therapies can be effectively readministered. Major challenges, however, include reduced efficiency of gene delivery to target cells and the often limited duration of expression of the therapeutic protein. Nonetheless, continued research in this area could lead to safe and cost-effective treatments for hemophilia.

Conclusions

Finally, one needs to be aware that the potential for immune responses to the therapeutic gene product, as it is the case in traditional factor replacement therapy, is a concern for development of novel gene therapies. However, some gene therapies, including hepatic gene transfer with AAV vectors, HSC gene transfer, platelet-derived expression, and others have been shown to induce immune tolerance or avoid responses to the expressed clotting factor [7,15,16]. Similarly, a number of immune modulatory protocols are being developed, which in combination with gene transfer could result in tolerance induction [18]. To conclude, these are exciting times for development of novel therapies for hemophilia, and scientists in the gene therapy arena are as determined as ever to find a cure and stop bleeding in these patients once and for all.

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