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REVIEW ARTICLE

Current status of haemophilia gene therapy

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Summary. After many reports of successful gene therapy studies in small and large animal models of haemophilia, we have, at last, seen the first signs of success in human patients. These very encouraging results have been achieved with the use of adeno-associated viral (AAV) vectors in patients with severe haemophilia B. Following on from these initial promising studies, there are now three ongoing trials of AAV-mediated gene transfer in haemophilia B all aiming to express the factor IX gene from the liver. Nevertheless, as discussed in the first section of this article, there are still a number of significant hurdles to overcome if haemophilia B gene therapy is to become more widely available. The second section of this article deals with the challenges relating to factor VIII gene transfer. While the recent results in haemophilia B are extremely encouraging, there is, as yet, no similar data for factor VIII gene therapy. It is widely accepted that this therapeutic target will be significantly more problematic for a variety of reasons including accommodating the larger factor VIII cDNA, achieving adequate levels of transgene expression and preventing the far more frequent complication of antifactor VIII immunity. In the final section of the article, the alternative approach of lentiviral vector-mediated gene transfer is discussed. While AAV-mediated approaches to transgene delivery have led the way in clinical haemophilia gene therapy, there are still a number of potential advantages of using an alternative delivery vehicle including the fact that *ex vivo* host cell transduction will avoid the likelihood of immune responses to the vector. Overall, these are exciting times for haemophilia gene therapy with the likelihood of further clinical successes in the near future.

Keywords: adeno-associated virus, gene therapy, haemophilia, lentivirus

Introduction

The clotting factor genes were among the earliest to be cloned in the early 1980s and as recessive traits, the haemophilias rapidly became targets for the application of somatic cell gene therapy. Over the past three decades, many strategies have been used to achieve persistent expression of therapeutically relevant levels of factor VIII (FVIII) and factor IX (FIX) in animal models of haemophilia. Indeed, there have been many successes of various gene transfer strategies with the long-term 'cure' of haemophilia A and B in mice and in smaller numbers of large animals. However, similar successes had not been documented in human disease until very recently.

In this State-of-the-Art review several key aspects of current haemophilia gene therapy science will be addressed. First, with the recent demonstration of gene therapy success in a very small number of haemophilia patients what are the challenges to the wider application of this therapy? Second, what distinct challenges are posed by FVIII gene transfer? And finally, what are the opportunities provided by an alternative gene transfer approach involving lentiviral vectors?

Challenges to the widespread application of haemophilia B gene transfer

With the report in late 2011 of successful long-term expression of Factor IX in six men with severe haemophilia B using intravenous administration of an AAV vector expressing the gene [1], the goal of gene

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therapy for haemophilia B would seem to have been reached. The following year, at the annual meeting of the American Society of Hematology, the same group reported successful treatment of four additional subjects at the highest dose. Yet the total number of people who have received this promising experimental therapy remains very low. What are the reasons for this low access rate to a seemingly successful new therapy?

Reasons related to scientific challenges in drug development

Prevalence of neutralizing antibodies to AAV in the adult population. From a scientific standpoint, even if men with severe haemophilia B were waiting for gene therapy in a line that stretched around the block, roughly 40% of individuals would still be excluded based on the presence of pre-existing neutralizing antibodies to AAV. Prior exposure to the wild-type virus from which the vector is engineered is ubiquitous in the population, and many individuals carry antibodies to the vector capsid. Population screening of individuals from four continents reveals that the worldwide prevalence of these antibodies is similar, and that, at least among most of the naturally occurring serotypes, the prevalence of antibodies is similar [2]. Careful studies in non-human primates, who, similar to the human population, carry pre-existing antibodies formed in response to infection with the wild-type virus, suggest that even modest titres completely inhibit transduction when vector is delivered through the circulation [3]. The field has proposed a number of strategies that could be used to circumvent this obstacle [4] (Table 1). At least one of these is currently undergoing clinical investigation [5], but proof-of-concept has not been established for any of these in subjects with severe haemophilia B.

Manufacturing considerations. Progress in manufacture of clinical grade AAV vectors since the first human studies were conducted in the 1990s has been dramatic, and most would now agree that methodology for generation, purification and characterization of recombinant AAV under current Good Manufacturing Practice (cGMP) conditions is well in hand. The products used in the clinical trials to date thus have met regulatory standards for safety, quality and consistency. However, most production methods currently in use lead to lot sizes in the range of $1–5 \times 10^{15}$ vg. Since a therapeutic dose for a 70 kg man based on current studies is $\sim 2 \times 10^{12}$ vg kg^{-1}, each lot is adequate to infuse 7–35 subjects, depending on yields. Thus, considerable attention is now focused on larger scale production processes. A commonly used manufacturing process involves transfection of a mammalian cell line, typically HEK 293 cells, with three plasmids, one encoding the AAV structural (*cap*) and replication (*rep*) genes, another the required adenoviral helper genes and a third the therapeutic gene of interest cloned between the two viral ITRs, with subsequent harvest of the recombinant virions and purification of these away from the other components of the manufacturing process, i.e. plasmid and cellular DNA and proteins. Using such a system, we produce approximately $1 \times 10^5–5 \times 10^5$ vg transfected cell^{-1}, with 10^{10} cells grown per week [24]. This process can be further scaled by moving from adherent cells to suspension culture for transfection; even allowing for some fall-off in vector productivity per cell on scale up, one can reach vector yields of 5×10^{12} to 1×10^{14} purified vg per litre of batch culture. An alternative production process relies on introduction of the required DNA components (same as above) into an insect cell line using expression vectors that are generated from baculovirus, a double-stranded DNA virus that naturally infects butterflies and moths. The baculovirus expression system has been reported to result in yields in the range of 7×10^{13} purified vg per litre of batch culture [25]. The products administered to subjects in the haemophilia trials to date have all been manufactured using the transient transfection mammalian cell culture systems. The sole licensed AAV product, Glybera for the treatment of lipoprotein lipase deficiency, was generated using a baculovirus system, and was administered by intramuscular injection. The concerns around the mammalian expression system include risks associated with residual plasmid or mammalian DNA impurities, and around the baculovirus system, the risks associated with residual xenogeneic (insect cell or baculoviral) DNA. The baculoviral production method has also been characterized by the generation of defective particles, which, if present, would increase the total capsid dose that must be delivered to achieve a set level of expression.

The human factor. At this point, there are three trials open and recruiting subjects for gene therapy for

Table 1. Possible strategies to overcome humoral immunity to AAV in systemic gene transfer.

Select subjects with low-to-undetectable anti-AAV NAb [6–8]
Administer higher vector doses [1,3,9–11]
Use empty capsids to adsorb anti-AAV antibodies thus allowing for vector transduction [11,12]
Administer immune suppression to prevent or eradicate humoral immune responses to AAV [13–17]
Switch AAV serotype [2] or engineer AAV capsids that are less susceptible to Nab [18–21]
Use repeated plasma exchange cycles to adsorb immunoglobulins and therefore reduce the anti-AAV antibody titre [22]
Use delivery techniques such as balloon catheters followed by saline flushing to isolate the target tissue from the systemic circulation to avoid vector dilution in blood and exposure to Nab [23]

Nab, neutralizing antibodies.

haemophilia B [26–28]. Moreover, two other groups have declared their intention of starting trials in haemophilia B [29,30]. Thus, opportunities to participate in trials would seem to be plentiful. Yet the pace of accrual to the ongoing studies seems slow. There are several reasons for this. First, most trials have mandatory pauses between subjects, to allow time to observe any adverse events before enrolment of the next set of subjects. Second, many interested participants at this point are still turned away, because they fail to meet the eligibility criteria of low or absent neutralizing antibodies to AAV (*vide supra*).

Manufacturing considerations are theoretically a limitation, although not until participation becomes more robust than currently. An additional factor, clearly though, is patient uncertainty about gene therapy. Gene therapy has had a chequered history, and in the view of the lay public may still be regarded as highly experimental. The reality to those engaged in the work is that, for AAV-mediated therapy, the short-term risks are well-delineated, consisting largely of an immune response that seems controllable by steroids, and a risk of lack of efficacy, if this can be considered a risk. However, it is also the case that direct clinical data addressing long-term risks of AAV-mediated gene transfer to liver is limited, since the total number of patient-years of follow-up is limited, and that the haemophilia community, based on the history of complications related to plasma-derived concentrates, is justified in expressing concern over perhaps unknown or poorly delineated long-term risks. The reality is that long-term follow-up of individuals with severe haemophilia B who were infused in the original trials from 2001 to 2004 has been reassuring [31], as has been long-term follow-up of >70 haemophilic dogs (Tim Nichols and Katherine High, unpublished data), and of normal non-human primates (Andrew Davidoff and Amit Nathwani, unpublished data) infused by the same route of administration. One important goal of all drug development is to provide patients with individual choices about how they manage their illness. The goal of the drug development process is to be able to label a product accurately in terms of risks that may be encountered. Until more long-term follow-up studies are completed in individuals who have received AAV-mediated gene therapy to liver, the level of certainty regarding long-term side effects will be lower than that with well-established recombinant protein products with longer treatment histories.

Gene therapy for haemophilia A using recombinant adeno-associated virus vectors:

Gene therapy for haemophilia A (HA), the most common severe inherited bleeding disorder, offers the potential of a cure through continuous endogenous expression of FVIII following a single therapeutic manoeuvre without significant toxicity. Haemophilia A is, in fact, well suited for a gene replacement approach because the disease phenotype is entirely attributable to the lack of a single gene product (FVIII) that normally circulates in minute amounts in the plasma (200 ng mL^{-1}). Importantly, a modest increase in the plasma FVIII levels to ≥1% of normal levels substantially ameliorates the bleeding diathesis and improves quality of life. Tightly regulated control of the FVIII gene expression is not required as a wide range of FVIII levels are likely to be efficacious and non-toxic. Liver-mediated FVIII expression may offer the additional advantage of induction of peripheral tolerance, thereby reducing the risk of inhibitor formation, which remains a major concern with protein replacement therapy. Finally, determination of the therapeutic end point can be readily assessed in most coagulation laboratories.

Several gene transfer strategies for FVIII replacement have already been evaluated in the clinic [32]. These include *ex vivo* electroporation of a FVIII expression cassette into fibroblasts, as well as intravenous delivery of a retroviral vector, or a helper-dependent adenoviral vector, for FVIII gene delivery. However, these trials failed to achieve persistent phenotypic correction in patients with severe HA [33,34]. Alternative strategies currently under investigation include the use of lentiviral vectors to transduce haematopoietic stem cells (HSC) or the liver [35]. Therapeutic FVIII expression has been achieved in HA knockout mice after transplantation of *ex vivo* lentiviral vector gene transfer of HSC with a hybrid human–porcine FVIII transgene [36]. In another elegant study, platelet-specific expression of human FVIII following *ex vivo* transduction of HSC with lentiviral vectors encoding FVIII under the control of a platelet-specific promoter resulted in effective haemostasis, even in animals with inhibitors, because of the ability of platelets to release the transgenic FVIII stored in platelet alpha granules locally at the site of injury [37–39].

We have focused our efforts on adeno-associated viral (AAV) vectors as they have an excellent safety profile and can mediate long-term transgene expression from postmitotic tissues such as the liver [40–42]. Indeed, our ongoing gene therapy clinical trial for haemophilia B, a related bleeding disorder, has demonstrated that a single peripheral vein administration of AAV vector leads to stable (>36 months) expression of human factor IX (FIX) at levels between 1 and 6% of normal [1]. This is sufficient for conversion of the haemophilia phenotype from severe to moderate or mild. More than two-thirds of the participants who were on prophylaxis prior to gene transfer have discontinued prophylaxis and remain free of spontaneous haemorrhage. The other participants have increased the interval between FIX prophylaxes.

The use of AAV vectors for HA gene therapy, however, poses new challenges due to the distinct molecular and biochemical properties of FVIII. Compared to other proteins of similar size, expression of FVIII is highly inefficient [43]. Bioengineering of the FVIII molecule has resulted in improvement of FVIII expression. For instance, deletion of the FVIII B domain, which is not required for cofactor activity, resulted in a 17-fold increase in mRNA levels over full-length wild-type FVIII and a 30% increase in secreted protein [44,45]. This has led to the development of B-domain deleted (BDD) FVIII protein concentrate, which is now widely used clinically (Refacto; Pfizer). Pipe and colleagues have shown that the inclusion of the proximal 226 amino acid portion of the B domain (FVIII-N6) that is rich in asparagine-linked oligosaccharides significantly increases expression over that achieved with BDD-FVIII [46]. This may be due to improved secretion of FVIII facilitated by the interaction of six N-linked glycosylation triplets within this region with the mannose-binding lectin, LMAN1, or a reduced tendency to evoke an unfolded protein response [47]. These six N-linked glycosylation consensus sequences (Asn-X-Thr/Ser) are highly conserved in B domains from different species suggesting that they play an important biological role [48].

Another obstacle to AAV-mediated gene transfer for HA gene therapy is the size of the FVIII coding sequence, which at 7.0 kb far exceeds the normal packaging capacity of AAV vectors. Packaging of large expression cassettes into AAV vectors has been reported but this is a highly inconsistent process resulting in low yields of vector particles with reduced infectivity [49,50]. AAV vectors encoding the BDD-FVIII variant that is around 4.4 kb in size show promising results using canine FVIII but further evaluation of this approach using human BDD-FVIII is required. Other approaches include the co-administration of two AAV vectors separately encoding the FVIII heavy- and light chains whose intracellular association *in vivo* leads to the formation of a functional molecule. The alternative two AAV vector approach exploits the tendency of these vectors to form head to tail concatamers. Therefore, by splitting the expression cassette such that one AAV vector contains a promoter and part of the coding sequence, as well as a splice donor site, whereas the other AAV vector contains the splice acceptor site and the remaining coding sequence. Following *in vivo* head to tail concatemerization a functional transcript is created that is capable of expressing full-length FVIII protein [51–53].

We have developed an AAV-based gene transfer approach that addresses both the size constraints and inefficient FVIII expression. Expression of human FVIII was improved 10-fold by re-organization of the wild-type cDNA of human FVIII according to the codon usage of highly expressed human genes [54–56]. Expression from B-domain-deleted codon optimized FVIII molecule was further enhanced by the inclusion of a 17 amino-acid peptide that contains the six N-linked glycosylation signals from the B domain required for efficient cellular processing. These changes have resulted in a novel 5.2 kb AAV expression cassette (AAV-HLP-codop-hFVIII-V3), which is efficiently packaged into recombinant AAV vectors and capable of mediating supraphysiological level of FVIII expression in animal models over the same dose range of AAV8 that proved to be efficacious in subjects with haemophilia B.

Summary

Our novel AAV-HLP-codop-hFVIII-V3 cassette substantially improves the prospects of safe and effective gene transfer for haemophilia A. We are currently in the process of developing clinical grade AAV-HLP-codop-hFVIII-V3 for use in human subjects in the context of a clinical trial, which we hope will open in early 2015. The design of this clinical trial will be discussed in greater detail during the meeting.

Progress in the use of recombinant retroviral vectors for the treatment of haemophilia A

Recombinant retroviruses used in clinical gene therapy applications have been extensively engineered for efficient transfer of nucleic acid sequences into human cells. The most significant modification is the creation of replication incompetent viruses. This means that whereas wild-type retroviruses produce viral particles after infection, replication incompetent retroviruses are devoid of sequences needed to replicate. Therefore, clinically used recombinant retroviruses have an engineered safety modification that only allows the transfer of therapeutic nucleic acid sequences into target cells, and the infected cell cannot generate additional viral particles. Nucleic acid sequences delivered by recombinant retroviruses are integrated into the genome of the targeted cell and can be transcribed for the life of that cell, as well as all of the progeny of the transduced cell.

Many retroviral vector-based strategies have been tested in preclinical models of haemophilia A, in both commercial and academic settings. There are several reasons for this interest. First, under optimal conditions gene transfer using recombinant retroviruses can be extremely efficient. Second, spontaneous bleeding can be alleviated by relatively low increases in FVIII levels, where as little as 2% normal levels can be beneficial. Third, although FVIII expression is generally considered to be liver specific, many studies have shown that different cell types are capable of synthesizing functional FVIII protein. Therefore,

virtually any cell type with access to the bloodstream can be targeted for gene transfer. With respect to retroviral gene transfer, the haematopoietic stem cell (HSC) is efficiently modified and transplanted, and has, therefore, been a reasonable target for haemophilia A gene therapy. Fourth, compared to repeated lifelong FVIII administration, retroviral-based gene therapy can be more economical because the number of treatment events should be limited, potentially to a single treatment. Fifth, because of the limited number of treatment events, gene therapy can be less invasive compared to protein replacement therapy that requires multiple weekly injections. The use of recombinant retroviral vectors is unique compared to other gene transfer technologies in that the transferred genetic material is integrated into the genome of the target cell, which can provide lifelong benefits. However, this benefit may be diminished by the potential adverse consequences of retroviral gene transfer.

The benefits and risks of gene transfer for haemophilia A compared to conventional intravenous replacement therapy have been discussed extensively [57–63]. It has been well documented that the principal concern with integrating viral-based gene therapy is the risk of insertional mutagenesis, which is the disregulation of endogenous gene functions as a result of the integrated nucleic acid sequence. The concern is based on initial retrovirus gene therapy studies where a T-cell leukaemia-like illness was found to be a serious adverse event observed in children enrolled in trials designed to treat the X-linked form of severe combined immune deficiency disease (SCID-X1) (reviewed in [64]). It was subsequently shown that γ-retroviruses, which were used in the initial trials, integrate preferentially near the promoters of active genes where the transcriptional enhancer of the transgene promoter has a higher probability of causing unregulated expression of proto-oncogenes. Therefore, we now know that under certain situations recombinant viruses can be oncogenic if they insert into the genome in the proximity of a gene that regulates cellular growth. As a consequence of this serious issue, clinical studies are now using gene-transfer systems based on lentiviral vectors. Lentiviral vectors, such as those derived from HIV-1, have multiple advantages compared to γ-retroviruses. Recent evidence shows that the use of advanced generation, self-inactivating recombinant lentiviral vectors for HSC gene transfer is safer than γ-retroviruses. It now is well documented that lentiviral vectors, unlike γ-retroviruses, do not integrate with high frequency near the promoters of proto-oncogenes and genes that control cell proliferation, and recent studies showed that they have a much lower oncogenic potential than other retroviruses. In addition, lentiviral vectors transduce HSCs as efficiently or, under some conditions, more efficiently than γ-retrovirus vectors.

The use of haematopoietic stem cells (HSCs) as the target cell population for lentiviral-mediated gene therapy applications is the most advanced application of this technology, and the use of lentiviral vectors for the treatment of haemophilia A has benefited from clinical trials that targeted HSCs for other genetic diseases. Because lentiviral-based gene transfer results in the genetic modification of the transduced cell's genome, the transduction process permanently modifies the DNA of the targeted cell. Bone marrow transplant studies in children have shown that transplanted HSCs survive for the lifetime of the recipient and that genetically engineered HSCs can both self replicate and/or differentiate into all cells of the haematopoietic system. In theory, transduction and transplantation of a single genetically modified HSC can result in the complete repopulation of the haematopoietic compartment, whereby all cells would be genetically modified. In the clinical setting, many diseases have already been treated using lentiviral-modified HSCs, including adrenoleukodystrophy, metachromatic leukodystrophy, Wiskott-Aldrich syndrome, chronic granulomatous disease, SCID-X1, HIV and thalassemia [65–71].

Based on encouraging clinical results using lentiviral vectors, preclinical studies using genetically engineered HSCs to treat haemophilia A are advancing towards clinical trials. Platelet-specific promoters have been used to treat both murine and canine models of haemophilia A. It is thought that this technology can be most useful in the setting of patients with pre-existing FVIII inhibitors. Lentiviral designs using promoters with more ubiquitous expression patterns have advanced to the stage of US FDA review. Although initial studies using recombinant retrovirus-modified HSCs showed limited FVIII expression in transplanted haemophilia A mice, recent studies using genetically modified FVIII sequences for enhanced FVIII expression combined with safety-enhanced recombinant lentiviruses have shown long-term therapeutic FVIII expression. A clinical trial using a self-inactivating lentiviral vector has been favourably reviewed by the US Recombinant DNA Advisory Committee as well as the US FDA in a preIND meeting, and funding for the clinical trial has been obtained through the US National Institutes of Health. It is, therefore, anticipated that a lentiviral gene therapy trial for haemophilia A is nearing clinical approval.

Disclosures

KH holds equity in and acts as a scientific advisor to, Spark Therapeutics. AN holds equity relating to several of his gene transfer products and has licensed some of these products to BioMarin. TS is the co-founder and holds equity in Expression Therapeutics. DL declares no conflicts of relevance to this text.

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