Gene therapy for haemophilia

Summary

The ultimate goal of gene therapy is the replacement of a defective gene sequence with a corrected version to eliminate disease for the lifetime of the patient. This challenging task is not yet accomplished, however significant progress is evident. An initial spate of clinical trials attempting the treatment of haemophilia with gene transfer primarily resulted in the demonstration of good safety profiles, but without efficacy. Subsequent reengineering of vector plasmids and delivery systems resulted in markedly improved outcomes in animal models of the disease. The most recent clinical trial for the treatment of haemophilia B with gene transfer showed transient achievement of efficacy in the highest dose cohort tested, but also exposed a previously hidden barrier to the future success of these treatments. The progress and problems of gene therapies for haemorrhagic disorders will be discussed. This review will concentrate on approaches in or near clinical application.

Keywords: haemophilia, gene therapy, vectors, adeno-associated virus.

Overview of gene therapy

The ambitious objective of gene therapy is to edit a defective gene sequence in situ to achieve complete reversion of a disease phenotype for the lifetime of the patient. In spite of recent successes in site-specific correction of defective gene sequences, the focus of most gene therapy strategies to date is on gene addition rather than gene replacement (Urnov et al, 2005). This simplified approach relies on a delivery mechanism to provide a corrected copy of the defective gene without removal of the error-containing genomic sequence.

While literally hundreds of animal models of disease can now be effectively treated by gene transfer, a select few diseases remain the primary focus of much gene therapy research. A combination of factors including prevalence of disease, width of therapeutic window, ability to accommodate the corrected gene sequence in a gene transfer vector, reliability and availability of animal models of the disease, and funding and support from disease-specific foundations, all contribute to the overrepresentation of these few diseases.

Haemophilia A and B are among the most extensively researched diseases in the field of gene therapy. Small and large animal models of both diseases are available for preclinical testing. Importantly, treatment of the disease can be quantitatively measured through well-defined coagulation assays, eliminating a problem that plagues gene therapy efforts for many other disease entities. Another important aspect of the treatment of haemophilia by gene transfer is that there is a relatively low threshold for success. If long-term expression of the defective coagulation factor at 2–3% of wild-type levels could be achieved, then a substantial reduction in the clinical manifestations of the disease would be expected (Herzog et al, 1999; Sarkar et al, 2000). Expression of greater than 30% of the wild-type level of the defective coagulation factor would result in a phenotypically normal patient under most circumstances (Pollak and High, 2001), although higher levels may be required in the face of haemostatic challenge (Plug et al, 2006).

Another advantage of haemophilia B in the development of gene therapy strategies is the relatively small size of F9 cDNA (~1.4 kB of coding sequence). This is amenable to insertion into many different gene transfer vectors and allows the addition of numerous transcriptional regulatory elements to both improve and restrict transgene expression in select cell types. F8 cDNA is much larger than that of F9 (>8 kB), and is not as readily accommodated in gene transfer vectors. Several strategies have been employed to overcome this difficulty. A first step towards more efficient packaging of F8 cDNA was deletion of the non-essential B-domain (Toole et al, 1986; Eaton et al, 1987). A dual-vector approach, in which the Factor VIII heavy chain and light chain are separately encoded by two different vector genomes, is another mechanism by which the
large transgene can be accommodated (Mah et al., 2003). A third technique, one that also employs two vectors, fragments two halves of a cDNA and tethers them through the use of a 3' splice donor site in vector genome A and a 5' splice acceptor site in vector genome B (Yan et al., 2000; Lai et al., 2005). In the future, improvements in vector manufacturing may make it possible to package even the larger F8 cDNA into the smaller gene transfer vectors without significantly compromising yield or homogeneity of vector preparations (Grieger & Samulski, 2005).

Early clinical trials for gene transfer treatment of haemophilia

Between 1998 and 2001 five different Phase I clinical trials were initiated for the treatment of haemophilia by gene transfer (Kay et al., 2000; Roth et al., 2001; Manno et al., 2003; Powell et al., 2003). Several different gene delivery systems were used in these trials, including a retroviral vector, an adenoviral vector, two different adeno-associated viral vectors, and a non-viral gene delivery method. The results from each of these trials are discussed here with background on each of the vector delivery systems.

Retroviruses are RNA viruses which use reverse transcription to generate a double-stranded DNA intermediate during replication. Replication-defective retroviral vectors also contain an RNA genome that is reverse transcribed and integrated into the host genomic DNA. Integration provides the potential for long-term, persistent gene expression but also increases the risk of the treatment through the potential for insertional mutagenesis and/or insertional activation of proximal genes, as was observed following retroviral transduction of haematopoietic cells in a gene therapy trial for X-linked severe combined immunodeficiency (Hacek-Bey-Ahina et al., 2003a,b; Fischer et al., 2004). Viral coding sequences are provided in trans during the manufacture of the vector and are not present at detectable levels in the vector itself. Many retroviruses, including murine leukaemia virus, are incapable of penetrating the nuclear membrane. These retroviruses and associated derivative viral vectors can only transduce dividing cells, requiring the natural breakdown of the nuclear membrane that occurs during cell division in order to enter the nucleus.

Given this limitation, it is not surprising that the most successful treatment strategies for liver-directed treatment of haemophilia using retroviral vectors devised strategies to induce hepatocyte cell division. One example of this was the use of a partial hepatectomy immediately preceding vector infusion (Kay et al., 1993). In this way, hepatocytes were induced to undergo cycling during the time of vector infusion. This approach only yielded low levels of Factor VIII expression (<1%), but partial correction for at least 5 months, in a canine model of haemophilia B. An alternative approach to target dividing hepatocytes is to infuse retroviral vectors into neonates, whose hepatocytes are naturally undergoing rapid cell division. This approach was successfully employed by VandenDriessche et al. (1999) to fully correct Factor VIII deficiency in a murine model of haemophilia A. Using an identical treatment in 13 neonatal mice, 8/13 mice demonstrated greater than 50% of wild-type Factor VIII activity by COATest (chromogenic Factor VIII activity test) assay. Long-term follow up revealed no significant loss of expression as long as 15 months after vector treatment. This approach was successfully extended to the canine model of haemophilia B by Xu et al. (2003), who achieved up to 3-5% of normal Factor IX activity levels following retroviral vector transduction of neonatal haemophilia B dogs.

Previously conducted preclinical studies led to the initiation of a phase I clinical trial testing a Moloney murine leukaemia virus-based retroviral vector encoding B-domain deleted Factor VIII for the treatment of haemophilia A (Greengard & Jolly, 1999; Roehl et al., 2000; McCormack et al., 2001; Powell et al., 2003). Doses ranging from 2.7 × 10^7 transducing units (TU)/kg to 4.4 × 10^7 TU/kg were tested. The treatment was well tolerated by all subjects and replication competent virus was undetectable in all of the collected samples. Factor VIII levels above 1% were sporadically detected but did not correlate with the dose administered. Vector DNA was detectable in peripheral blood mononuclear cells (PBMCs) in 4/8 subjects that received 2.8 × 10^7 TU/kg and 8/8 subjects that received 9.2 × 10^7 TU/kg up to 53 weeks after the infusion. Overall, the signs of clinical improvement following vector infusion were modest at best. These findings were consistent with animal studies suggesting that efficient retroviral transduction of hepatocytes would require higher doses and some degree of mitotic induction.

Adenoviral vectors have been frequently used in preclinical gene transfer studies of haemophilia. Advantages of these double-stranded DNA vectors are high transgene expression levels and the ability to transduce hepatocytes efficiently in vivo. Frequently, however, expression from early generation vectors was transient, owing to the immunogenic properties of the adenoviral vectors themselves. In contrast to other vectors that are devoid of viral gene sequences, early generation adenoviral vectors encode many viral proteins in addition to the transgene; these may contribute to the immunogenicity of these vectors (Schagen et al., 2004). In mice, the immune response was not sufficient to ablate the effects of the treatment and curative levels of Factor VIII or Factor IX could be achieved and sustained for 3–5 months in murine models of Haemophilia A and B (Smith et al., 1993; Connelly et al., 1996; Walter et al., 1996). In contrast, large animal studies demonstrated that the duration of transgene expression was significantly shorter than that seen in mice and that hepatotoxicity was correspondingly greater (Kay et al., 1994; Lozier et al., 1999). In a canine study, a vector dose of 2.4 × 10^12 plaque forming units (pfu) infused into three haemophilia B dogs resulted in transient expression of Factor IX at supraphysiological levels. These levels rapidly declined, reaching 1% of normal by 3 weeks after vector infusion and
0·1% 2 months postvector infusion. A comparable dose of the same vector in mice resulted in sustained levels of Factor IX expression at 20% of normal for over 4 months (Kay et al, 1994). Further substantiating these findings was a non-human primate study in which an adenovirus vector was used to deliver a human Factor IX transgene. In this study, a peak of 80% wild-type Factor IX levels was achieved at the highest dose (1 × 10¹¹ pfu/kg) followed by a rapid decline to baseline, with human Factor IX levels undetectable by 3 weeks after vector infusion (Lozier et al, 1999). Moreover, significant hepatotoxicity was observed. Serum aspartate transaminase (AST) levels rose above 500 U/ml at both the intermediate dose and the high dose, and bilirubin levels rose above 85·5 μmol/l in the high dose cohort only (Lozier et al, 1999). Thrombocytopenia was also observed in the intermediate dose and high dose cohorts. Thrombocytopenia was subsequently linked to decreased fibrinogen levels and increased platelet clearance time believed to result from the hepatotoxicity of the vector (Lozier et al, 1999; Wolins et al, 2003). Recent work by Othman et al (2007) showed that activation of platelets occurs following exposure to adenovirus. Activation is, in turn, followed by upregulation of P-selectin, which is known to promote platelet clearance (Othman et al, 2007). They also identified a critical role for von Willebrand factor (VWF) in the induction of thrombocytopenia following adenovirus infusion. Using a vector dose that results in thrombocytopenia in wild-type mice (1 × 10¹¹ pfu), significant thrombocyte- nia was not observed in VWF knockout mice.

Further development of adenoviral vector production systems allowed complete elimination of viral coding sequences from the vector. The intent of this manoeuvre was to minimize the immunogenicity of these vectors. Using these ‘gutted’ adenoviral vectors, sustained correction of disease phenotype was observed in haemophilia A mice infused with 3 × 10¹² vector particles (vp)/kg with no apparent hepatotoxicity. Non-human primate studies showed some dose-dependent hepatotoxicity with the threshold residing between 1·4 × 10¹² vp/kg and 4·3 × 10¹² vp/kg. A Phase I clinical trial was initiated to test the safety of an adenoviral vector encoding human Factor VIII for the treatment of Haemophilia A; because of a priori concerns regarding immunogenicity, the trial was structured to monitor carefully for changes in liver function tests or platelet count. The first subject, enrolled at the lowest dose of 4·3 × 10¹⁰ vp/kg, experienced inflammation, fever and myalgia upon vector infusion; these symptoms are commonly observed with infusion of adenoviral vectors. The subject also experienced thrombocytopenia and an elevation in serum transaminases that peaked 7 d after infusion and returned to baseline by 19 d postinfusion (Chua et al, 2004). Due to safety concerns and a perceived narrow therapeutic index, no additional subjects were enrolled in this trial.

A third vector modality used in this first group of clinical trials was the adeno-associated virus (AAV) vector. AAV is a small, single-stranded DNA virus that is naturally replica-
vector. Accordingly, more intramuscular injections of the vector would be required, and the number of injections thought to be required to reach a therapeutic effect was deemed clinically impractical.

In addition to viral vector delivery methods, one non-viral delivery method was also tested in a clinical trial (Roth et al, 2001). This approach, similar in some respects to previous clinical studies conducted in China (Lu et al, 1993; Qiu et al, 1996), consisted of transplantation of F8-transduced autologous fibroblasts. After isolation from a skin biopsy, patient cells were transfected with a plasmid encoding a human F8 cDNA ex vivo and stable selection for transfectants was carried out. Single clones were expanded and tested for Factor VIII expression level, as well as tumorigenicity and microbial safety, prior to reimplantation onto the omentum. Animal model studies were promising for this approach, but data acquired from the Phase I clinical trial showed only a modest and temporary indication of positive effects. The treatment was, however, well tolerated and leaves open the possibility of future attempts using more potent expression systems for the ex vivo transduction and selection process. An important step in advancing this treatment modality will be the determination of the cause for the apparent loss of expression over time. Possible obstacles to durable transgene expression include: senescence of the implanted cells, promoter inactivation, fibrosis around the transplanted cells and immune responses to the gene-modified cells.

Recent developments in gene transfer for haemophilia

Adenovirus vectors are still being pursued as a means of obtaining long-term expression of both Factor VIII and Factor IX. Improved liver-specific promoters and further redesign of production methods resulted in long-term expression of Factor VIII in canine models of haemophilia A, although some hepatotoxicity remains evident and could complicate translation into clinical trials (Andrews et al, 2002; Chuah et al, 2003; Brown et al, 2004). Specifically, inter-subject variation and a small therapeutic index make the safe use of adenoviral vectors difficult for stable gene transfer in humans. As a result of their inherent immunogenicity, adenoviral vectors are now more frequently used as vaccine delivery vehicles (Tatsis & Ertl, 2004).

New developments in the field of retroviral vectors are more promising for application in the treatment of haematological disorders. One of the most important innovations has been the development of lentiviral vectors, which have several advantages over the first-generation retroviral vectors. First, they are capable of transducing non-dividing cells, making them more suitable for transduction of, for example, hepatocytes and haematopoietic stem cells. Second, while retroviral vectors preferentially integrate their genomes near transcriptional start sites, lentiviral vectors show a random integration pattern into the open-reading frames of genes (Mitchell et al, 2004). While this difference does not eliminate the risk of insertional mutagenesis, it seems likely to mitigate the risk by reducing the number of full length gene transcripts that might be activated through vector genome insertion. Improvements in insulator elements flanking coding sequences within the vector genome itself further reduced the potential for undesirable insertional activation events (Chung et al, 1997). Naldini et al showed that the use of a liver-specific promoter in place of a CMV promoter could alone be a determinant of stable lentiviral transduction of hepatocytes (Follenzi et al, 2004). With a ubiquitous CMV promoter driving expression of either green fluorescent protein (GFP) or human Factor IX, expression was short-lived and the loss of expression was accompanied by both antibody formation against the transgene and T cell infiltrates in the liver. In contrast, use of a liver-specific promoter resulted in long-term, stable expression of GFP or human Factor IX in wild-type mice with expression levels of the latter reaching 200 ng/ml. In a subsequent study, Naldini et al also showed that the incorporation of four copies of a microRNA target sequence in the vector genome could selectively mark vector transcripts for destruction in cells expressing the corresponding microRNA (Brown et al, 2006). Using this lineage-specific suppression strategy, selective downregulation of transgene expression in hematopoietic cells was achieved. This vector demonstrated decreased immunogenicity and more robust, stable transgene expression in immunocompetent mice.

Silencing elements encoded by a lentiviral vector transcript were also used to demonstrate therapeutic effect in a recent study of lentiviral-mediated gene transfer for the treatment of sickle-cell disease. Unlike many other genetic disorders, sickle cell disease cannot be completely corrected by the addition of a wild-type copy of the defective gene. The mutant globin would act as a dominant negative gene product and suppress the activity of the wild-type therapeutic globin. Similar to the approach taken by Brown et al (2006), Samakoglou et al (2006) developed a small interfering RNA (siRNA) element targeting sickle-globin RNA that was inactive against the vector-encoded therapeutic globin RNA. The siRNA was incorporated into an intron within the gamma-globin transgene. Following transcription of the transgene, both expression of the therapeutic gamma-globin and post-transcriptional downregulation of the beta-globin (sickle) RNA levels were observed. This experiment was conducted in haematopoietic cells taken from human patients with sickle cell disease, and provides proof-of-principle that this approach could be translated into the clinic. Chang et al (2006) recently demonstrated the potential for lentiviral transduction of haematopoietic stem cells for the production of Factor IX for the treatment of haemophilia B.

Continued advances were also made in animal models of AAV vector-mediated gene transfer for treatment of haemophilia. Substantial improvements in transgene expression levels were made through the use of liver-specific promoters and portal vein administration. Therapeutic levels of human Factor...
AAV vector transduction in the liver can result in the either at the time of administration or at later times. In fact, neither AAV2 nor any of the novel serotypes have date, neither AAV2 nor any of the novel serotypes have resulting in 10–100\(\times\) serotypes was far superior transduction efficiency in the liver, application. Yet another advantage seen with the novel AAV8 route of administration, however, this finding did not ascend the evolutionary ladder. Non-human primate studies directly the observation confined to a single subject, the chance of a explanation for the loss of transgene expression was a T-cell protocol included to allow assessment of T cell responses to the transduced cells. This phenomenon had not been observed in any animal model of AAV transduction, but unlike the animal models, humans are frequently exposed to wild-type AAV viruses in the context of pathogens from very early childhood (Blacklow et al., 1968; 1971). These exposures, because they occur in the context of helper viruses, such as adenovirus that evoke a strong immune response, may also evoke an immune response to AAV vector proteins that could be reactivated by AAV vector infusion. Without serial PBMC collection, this hypothesis was difficult to test, and with the observation confined to a single subject, the chance of a repeat occurrence of this sequence of events was unclear.

Consequently, another subject (subject G) was enrolled at the intermediate dose (4 \(\times\) 10\(^{11}\) vg/kg) with a PBMC collection protocol included to allow assessment of T cell responses to Factor IX and to AAV. The results of this analysis convincingly demonstrated that a T-cell response to the AAV vector capsid protein occurred following vector administration. The subject’s T-cell responses were monitored during this time by interferon-gamma enzyme-linked immunosorbent assay (ELISpot), using pools of 15 amino acid peptides overlapping by 10 amino acids that spanned the entire vector capsid sequence and the entire F9 sequence. Although T-cell responses to the vector capsid were undetectable at baseline,
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responses significantly above background were readily detected by the first PBMC collection time point, 2 weeks after vector infusion, and remained positive at each time point tested up to 12 weeks after vector infusion. Simultaneous ELISpot assays to detect immune responses to the Factor IX protein were consistently negative. In the 14 weeks following vector administration, subject G experienced a mild transaminitis (peak AST and ALT levels of 67 IU/l and 105 IU/l respectively) with a temporal course closely matched to that experienced by subject E (Manno et al., 2006). As with the other two subjects infused at this dose, no Factor IX activity levels >1% were detected following infusion of the vector. These findings strongly implicate a T cell response to AAV capsid in the loss of Factor IX expression in the subject who transiently expressed therapeutic levels of the transgene.

Epitope mapping using the results from the ELISpot assays identified a nine amino acid peptide that appeared to be an immunodominant epitope in the anti-capsid T-cell response of subject G. This knowledge was used to generate a pentamer reagent that enabled direct detection of the capsid-specific CD8$^+$ T-cell population responding to this epitope. The kinetics of the T-cell response in subject G were mapped with this reagent and shown to overlap with the peak of serum transaminases (Mingozzi et al., 2007). Two years after vector infusion, PBMCs were isolated from subject E. No T-cell response to AAV capsid was detectable directly ex vivo. Upon expansion in the presence of AAV capsid peptides, however, a single immunodominant epitope from the AAV capsid invoked a significant expansion of T cells from the PBMCs of subject E. Following two rounds of PBMC expansion in the presence of this peptide, 8.8% of the CD8$^+$ T cells in this culture secreted interferon gamma upon re-exposure to the epitope (Mingozzi et al., 2007). Also of note from these studies, immune responses to AAV capsid were detectable in normal, uninfused subjects in both PBMCs and splenocytes, suggesting that the events seen in this trial were not aberrant and would be repeated if additional donors were infused.

Given the likelihood that an immune response to AAV capsid ablated the transduced cells, and that capsid is present only transiently before being degraded and cleared from the cells, one potential solution is to block the immune response to capsid pharmacologically until capsid has been metabolized and cleared from the cells. This raises the question of the duration of persistence of the capsid in the transduced cell, or more precisely, the duration of persistence of peptide-major histocompatibility complexes on the cell surface. Attempts to generate an animal model that replicates these findings have so far been unsuccessful (Li et al., 2007a,b; Wang et al., 2007). Most approaches have relied on a prime-boost regimen with adenoviral or plasmid vectors expressing AAV capsid, to generate a robust CD8$^+$ T cell response to AAV capsid. However, when these immunized mice are challenged with an AAV vector encoding a human F9 transgene under the control of a liver-specific promoter (Li et al., 2007b), the transduced hepatocytes persist even in the presence of AAV capsid-specific T-cells. In fact, no diminution in transgene expression was detected as compared to unimmunized mice or control immunized mice.

The next phase in gene transfer for haemophilia

Safe, long-term expression of clotting factors has been successfully achieved in large animal models of haemophilia using multiple gene transfer strategies, but these findings have not yet been translated into success in patients. Ongoing and proposed clinical studies should help to determine whether AAV-mediated gene transfer to the liver can achieve success. The initial liver trial is set to resume, at a dose approximately half that used in subject E, and with the addition of transient immunosuppression to block the T-cell response to the capsid. If a capsid-specific T-cell response was indeed causative in the loss of transduced hepatocytes in subject E, an important question remains: will the immunosuppressive regimen selected be sufficient to block the response? It is also not entirely clear how long the regimen will need to be maintained. Alternative hypotheses to explain the findings in the liver-directed AAV trial have been proposed. One hypothesis suggests that fragments of the AAV capsid were expressed in the transduced cells as a result of low-level packaging of these sequences during the vector production process (http://www4.od.nih.gov/oba/RAC/meeting.html). This hypothesis does not adequately explain the difference in findings between animal models and humans. A second hypothesis is that an alternate open reading frame contained within the F9 cDNA encodes an immunogenic protein sequence, but this hypothesis also fails to explain the difference between animals and humans (http://www4.od.nih.gov/oba/RAC/meeting.html). Other hypotheses focus on differences in the sensitivity of immune responses in animals and humans. There is some evidence that T cells in humans may be more sensitive than T cells in non-human primates as a result of differential expression of an immunomodulatory lectin (Nguyen et al., 2006). Yet another possibility is that a low level of CpG methylated DNA is packaged into the vector during production and that humans possess more sensitive innate immune responses to these stimuli. Finally it has been proposed that there will be differences in capsid processing and presentation with alternate AAV serotypes, because of different kinetics of uncoating, differences in intracellular trafficking, or differences in antigen processing (Vandenbergh et al., 2006). Based on strong preclinical data in non-human primates, a proposed trial of AAV8 encoding a F9 transgene will test this hypothesis (Nathwani et al., 2006). This next-generation vector carries a self-complementary expression cassette to enhance expression at lower doses and encodes a codon-optimized transgene to improve translational efficiency. Will the muscle ultimately be a better target for AAV-mediated Factor IX expression? It is clear that Factor IX transgene expression persisted in subjects injected with AAV vectors intramuscularly. New systemic approaches to introduce AAV vector into skeletal muscle have since been
developed (Arruda et al, 2005). Will these delivery methods result in stable expression or transient expression of the transgene? Continuing studies should provide answers to these questions, and ultimately a safe and effective long-term treatment for haemophilia.

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