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GENE THERAPY FOR THE HEMOPHILIAS

Revised Edition

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Gene Therapy for the Hemophilias

David Lillicrap, Arthur R. Thompson

Summary

Clinical trials of gene transfer in the hemophilias evolved from a decade of studies in cultured cells and then in animal models. The limited success from the initial human experience has provided focus on a need for improvements in or alternatives to the viral vector transfer systems used in subjects with hemophilia A or B. Several laboratories have modified vectors to improve expression of these clotting factors, while considerable effort is aimed at improving safety. Although a variety of new approaches are promising, a clearly improved strategy has yet to emerge.

Incorporation of intron and other elements into the factor VIII or IX transgene (cDNA) improves the amount of factor expression in each cell. Tissue specific promoters limit expression in other tissues including immune cells, to reduce the risk of developing inhibitors. Retroviral vectors, the ones originally introduced for gene transfer, are more successful in neonatal mice; the related lentiviral vectors will transduce non-dividing cells, although the long-term safety of either remains a concern. Adenoviral vectors stripped of their viral genes have fewer complications, but there is still the need to develop a safer delivery envelope. Different serotypes and modified adeno-associated viral vectors increase expression and allow inclusion of the larger factor VIII genes. Effective delivery systems for non-viral vectors in vivo *require developing; nevertheless, a rodent model demonstrates that long-term expression of therapeutic levels of factors VIII or IX can be realized without a viral vector. Although the primary target cell for gene transfer remains the liver, there is progress with expression from a variety of other tissues. Strategies for tolerance induction or prevention of immune responses to expressed factors VIII and IX are emerging to improve the safety profile and developments in understanding the immune response, especially to factor VIII, are addressing this potentially severe complication. Finally, efforts of gene repair are showing initial progress, especially with a "trans-splicing" approach that corrects the host's own mutant messenger RNA, allowing synthesis of a normal protein even in the absence of the normal gene sequence.*

Introduction

For a patient with hemophilia, gene therapy would allow continuous synthesis of a normal protein to correct the deficiency state *in vivo*. The resultant protein synthesis would be, in one sense, comparable to a "cure" as observed in the uncommon hemophilic patient that has received liver transplantation. A distinction with organ transplantation is that in gene therapy, one's own cells are transduced, the process of adding a functional gene to a cell. Thus immuno-suppression and its toxicity (as required for transplanted organs to prevent rejection) is not necessary. Initial studies led to considerable optimism that hemophilia could be cured (Verma, 1990). The basic elements of gene transfer, including different vectors, delivery systems, and target cells used with factors VIII or IX have been reviewed (High, 2003; VandenDriessche, 2003; Walsh, 2003).

A distinction should be drawn between gene therapy to modify a host's somatic cells and other highly experimental approaches that lead to transgenic animals. Such animals (e.g., "Dolly") are capable of passing on the genetic modification to the next generation as it involves all of their cells, including those of their germline (eggs and sperm). Transgenic sheep were created to synthesize human factor IX and contained the human factor IX DNA in all tissues (Schnieke et al, 1997). This approach may be useful in generating female animals such as pigs that secrete human factors VIII or IX in their milk (Paleyanda et al, 1997; Van Cott et al, 1999).

A second distinction is between gene transfer and gene repair. All of the human and most of the preclinical animal studies have focused on gene transfer where extra copies of the normal coding DNA (cDNAs) for factors VIII or IX are inserted into cells that were previously unable to synthesize a normal clotting factor. For gene repair, relatively limited success has been realized to date in either cultured cells or animal models although there are some promising,

novel approaches being developed that will likely be worthy of trials in animals.

Animal models of hemophilia gene transfer with viral-derived vectors have been the most reliable at providing sustained therapeutic levels of the clotting factors. These animal models include mutations that have occurred naturally in dogs (then bred to form hemophilic colonies) or in mice made factor VIII or IX deficient by targeted gene disruption, so-called "gene knockout" mice (Rawle and Lillicrap, 2004).

Vectors for Gene Transfer

The transfer of DNA into cells for gene therapy is accomplished by transduction, a controlled process that is mediated by vectors or vehicles that attach to a cell surface and facilitate entry into the cell. This is in contrast to transfection where cell membranes are disrupted and DNA insertion occurs by physical or electrochemical means. Vectors for transduction are commonly derived from viral nucleic acid backbones as these are more efficient than current non-viral preparations. The portion of the virus that directs cell attachment and entry is retained. Vector systems with promising preclinical animal studies relevant to gene therapy for the hemophilias are summarized in Table 1. A final section of this review highlights considerations for their use in clinical trials.

Retroviral vectors

An early strategy in gene therapy was to use retroviral vectors to transduce dividing cells in tissue culture and return the transduced cells to the host animal by transplantation (Van Damme et al, 2004). However, transgene expression levels in a variety of cell types were considerably higher in culture than *in vivo* secondary to gene silencing after transplantation. These problems led to efforts to improve methods to administer vectors directly to animals and transduce the cells *in vivo*. The principle of long-term (sustained) expression following *in vivo* administration of a retroviral vector was established when hemophilia B dogs expressed normal canine factor IX for over two years, although levels were insufficient to correct the bleeding tendency. Furthermore, because of the retroviral vector's requirement for dividing cells, these dogs underwent partial hepatectomy in order to increase the number of cells in division at the time of portal vein infusion of the viral construct. Other strategies used to generate sustained in

Table 1: Viral Vectors Used for Preclinical Gene Transfer of Factors VIII or IX

Vector	Nucleic Acid	Advantages	Limitations	Reference
Retroviral	RNA	• efficient transduction • genomic integration • persistent expression	• oncogene derivation • random insertion • cell division-dependent (except lentiviral)	Van Damme et al, 2004
Adenoviral	DNA	• transduces non-dividing cells • accommodate large cDNAs • high level of expression	• immune responses to AV • episomal (no integration) • transient expression	Thorrez et al, 2004
Adeno-Associated Viral	DNA	• integration (partial) • persistent expression • different serotypes	• limited size of cDNA • possible rearrangements	Couto, 2004

vivo factor IX expression from retroviral vectors include gene transfer into neonatal hosts or into the livers of adult animals stimulated by growth factors to induce hepatocyte proliferation.

Concerning factor VIII, rabbits displayed a delayed increase in expression of human factor VIII when the B domain-deleted cDNA was given intravenously in a retroviral vector. Expression persisted for at least several weeks, although activity could not be assessed due to the presence of neutralizing rabbit anti-human factor VIII antibodies. Because of a limitation of size of the sequence inserted into retroviral vectors, B domain-deleted factor VIII constructs have been used as these are only about two-thirds the size of the full length cDNA. The degree to which immune responses in animals, including hemophilia A dogs, reflects the use of transgenes from another species (human factor VIII, for example) versus an alloimmune response to factor VIII itself needs clarification.

There have been attempts to use different retroviral vectors that will transduce resting as well as dividing cells to improve the efficiency of transduction. Lentiviruses are a class of retroviruses that are able to infect resting cells. The most widely studied lentiviral vectors are based on the human immunodeficiency virus (HIV). These are more complex retroviral derivatives that are capable of penetrating the nuclear membrane and can therefore integrate into the cell's DNA at any stage of the cell cycle. Initial studies on the safety and efficacy of these vectors in mouse models of hemophilia have shown promise (Van Damme et al, 2004; Follenzi et al, 2004).

Adenoviral vectors

Adenoviral vectors have the advantage of being able to incorporate large DNA fragments into viral DNA sequences rendered non-pathogenic by partial deletions of the viral genome. They also readily transduce cells with large numbers of viral particles and infect both resting and dividing cells. However, they still express several viral proteins that are immunogenic, leading to more rapid clearance of the vector and transduced cells. Furthermore, repeat transduction is usually not successful, unless the initial exposure was accompanied with immune modulation to suppress an initial response to adenoviral capsid (coating) proteins. Repeat transduction is particularly important as the adenovirus or adenoviral vectors remain episomal within the nucleus, i.e., not integrated into the host chromosomal DNA. There has been success in animal models with prolonged expression of factor VIII or factor IX (Thorrez et al, 2004), particularly using constructs that contain disruptions in several adenoviral genes and when vectors are administered to host animals in which the immune response has been suppressed. Further success has been achieved with a type of adenoviral vector in which all the adenoviral genes have been removed (gutless, helper-dependent or "mini-Ad" vectors). Delivery of these constructs into hemophilic mice and dogs has resulted in therapeutic expression of clotting factor levels for up to several months (Chuah et al, 2003; Brown et al, 2004). In these latest generation adenoviral vectors there is no production of viral proteins that would enhance vector clearance from cells. However, there are still acute, transient adverse effects of adenoviral vector delivery including thrombocytopenia and damage to liver cells. Multiple administrations without host immune modulation would still be problematic due to the immune response against the vector capsid proteins. Nevertheless, because there is still no vector integration, the expressed levels of protein diminish over the course of a few weeks to several months, making it likely that repeat administration of adenoviral vectors would become necessary at some point.

Adeno-associated viral (AAV) vectors

AAV vectors are able to transduce a variety of dividing and non-dividing cell types and persist within the nucleus of these cells for prolonged periods of time (Couto, 2004). Most of the input vector remains in a stable extra-chromosomal form, but a small proportion of the vector (approximately 5%) appears to integrate into the host cell genome. When AAV vectors are given to "permissive" strains of mice (those with a low potential to develop antibodies to the human protein) bred to other mice with a factor IX gene knockout, expression of therapeutic levels of human factor IX has been sustained for several months. Phenotypic correction (no bleeding) and expression in these initial factor IX knockout mice has persisted at 15% levels for over one year; furthermore, with a higher vector dose, factor IX levels approaching 100% have been achieved. Hepatocytes can also be

transduced by infusion of AAV vectors into peripheral veins although a portal vein injection (the vein that flows directly from the intestinal circulation to the liver) provides more efficient transduction. On administration of an AAV vector carrying the canine factor IX cDNA to dogs with severe hemophilia B, either by multiple intramuscular injections or infused into the portal vein, expression of therapeutic levels of factor IX for longer than five years has been obtained. A canine B domain-deleted factor VIII cDNA has also been inserted into an AAV vector and portal vein injection of this construct has resulted in expression of therapeutic levels of factor VIII in hemophilic dogs and mice (Scallan et al, 2003a; Scallan et al, 2003b).

Non-viral vectors

The advantage of a non-viral delivery system is to avoid potential toxicities of viral vectors be it from integration or inflammatory and/or immune responses to viral capsid proteins. As non-integrating systems, however, one would not anticipate prolonged expression. Furthermore, a major difficulty is stabilizing the DNA and efficiently inserting it into cells *in vivo* (Gomez-Vargas & Hortelano, 2004). There has been surprisingly long-term expression of factors IX or VIII, however, in immuno-deficient hemophilic mice suggesting that episomal expression can persist providing one has a highly efficient vector construct. In factor VIII deficient normal mice, however, alloimmunization occurred even when a species specific murine factor VIII was used (Ye et al, 2004). The degree to which the immune response is influenced by initially high levels of gene expression and/or cytokine responses evoked during delivery remains to be assessed. A novel alternative approach is to use non-viral vectors containing transposons derived from ancestral genetic sequences. Vectors with transposons can integrate into host cell genomic DNA if delivered concomitantly with the transposase enzyme. Recently, it was demonstrated that a transposon vector containing the factor IX cDNA was able to integrate into mouse liver cells and direct long-term gene expression and partial phenotypic correction of hemophilia B mice. (Mikkelsen et al, 2003).

Alternative cell types

The hepatocyte is well established as the site of synthesis of factor IX and the liver is the primary organ of synthesis of factor VIII. There may well be contribution of both hepatocytes and sinusoidal endothelial cells for the latter. Blood outgrowth endothelial cells can be harvested, transduced *in vitro* with factor VIII and serve as a source for gene transfer (Lin et al, 2002). Hematopoietic cells have long been a target due to the accessibility of stem cells in peripheral blood. Although yields have been disappointingly low, there is early success with factor VIII using a lentiviral vector (Tiede et al, 2003). Megakaryocyte expression of factor VIII would have a potential advantage of co-expression of the stabilizing von Willebrand factor protein. This has been accomplished in cultured cells using a megakaryocyte-specific promoter (Shi et al, 2003).

Gene repair

RNA/DNA hybrid oligonucleotides have been used to correct point mutations (Kren et al, 1998). Although limited success has been reported in some animal systems, efficiency, particularly *in vivo,* is a major problem with this approach. There is some evidence that nonsense mutations can be transiently suppressed at the ribosomal level using aminoglycoside antibiotics such as gentamycin. This could potentially convert severe hemophilia to a moderately severe phenotype in selected patients with this type of hemophilic mutation. Use in 5 hemophilic subjects with nonsense genotypes, however, failed to show a clinically significant effect (James et al, 2003). Trans-splicing is another mechanism for gene repair at the RNA level, and could potentially be applicable to inversions; early studies with factor VIII in mice suggest that it may be a feasible approach for the hemophilias (Chao et al, 2003) and, if successful, would avoid the potential toxicities of viral vector gene transfer. Optimal delivery systems for the latter remain to be developed.

Human Trials of Hemophilia Gene Transfer

As vector systems capable of delivering the factor IX or VIII cDNAs to host cells have been optimized to express sufficient quantities of the clotting proteins *in vivo*, an important issue has been the design of therapeutic trials in human patients. Issues of safety are paramount in assessing the potential risks versus the possibility of making hemophilia less severe or enacting a

long-term cure. In the following sections, the results of initial human clinical trials of hemophilia gene therapy will be summarized and potential safety concerns are discussed for each vector system followed by considerations as to which patients represent appropriate candidates for these initial human studies.

To date, five clinical trials of hemophilia gene therapy have been completed involving a total of 41 patients (Table 2). All of these studies have been phase I/II trials in which the primary purpose has been to assess potential toxicity of treatment (phase I) and to determine if there are any early signs of therapeutic benefit (phase II). It should be noted that no patient showed any evidence of inhibitor development as a response to gene transfer. None of the patients treated in these trials experienced significant long-term adverse effects but two individuals had transient side effects related to their treatment with viral vectors.

Hemophilia A

There have been three trials of factor VIII gene therapy given to 26 subjects with hemophilia A. Two of these used viral vectors delivered *in vivo*, whereas a third used non-viral delivery to cultured, autologous cells (Chuah et al, 2004).

In a non-viral vector system, autologous fibroblasts were harvested from a skin biopsy and during culture were transfected by electroporation with the cDNA of a B domain-deleted factor VIII gene containing a fibronectin promoter to limit the potential for expression in other cell types (Roth et al, 2001). B domain-deleted factor VIII protein is commercially available as harvested from cultured, transfected cells and appears comparable in recovery, survival, and its clinical effect for treatment or prevention of bleeding to full-length ("wild-type"; B domain-included) factor VIII. The fibroblasts from these subjects were then selected for their ability to express factor VIII and secrete it into the culture media. These cells were then implanted laparoscopically into the peritoneal cavity by injection into omental fat pads. Patients were given factor VIII concentrate to prevent bleeding associated with the invasive procedure. Only minor adverse events were encountered such that the procedure appears quite safe and no long-term complications occurred. Post-therapy, clotting factor levels were measured and in some were at or slightly above the 1% limit of detection for the clotting activity assay. Within several days to a few weeks, however, all levels returned to pre-treatment baselines. Another potential benefit seen in some of these subjects was a reduction of spontaneous bleeding episodes that required exogenous infusions of factor VIII concentrates. However, it has to be recognized that this latter outcome may be difficult to distinguish from a placebo effect.

Another factor VIII gene transfer trial used an early generation retroviral vector derived from a murine leukemia virus (Powell et al, 2003). The vector contained the viral promoter sequence to express a B domain-deleted factor VIII. It was administered by simple intravenous infusions on three successive days. The vector infusions and subsequent courses were well tolerated with no complications noted. Post-therapy clotting factor VIII clotting activities, although somewhat variable as in the fibroblast study, showed no consistent elevations above the 1% limit of detection. Furthermore, there was no dose-response seen. Within several days to a few weeks, all activities were at pre-treatment levels. However, again some subjects reported fewer spontaneous bleeding episodes in the weeks following gene transfer. Although data were limited, there may have been a prolonged survival to transfused factor VIII that would suggest a low (albeit undetectable by routine assays) level of factor VIII circulating.

An adenoviral vector that was “helper dependent”, that is, stripped of its viral genes, and with the full-length factor VIII cDNA inserted, was administered intravenously to one patient (White, 2003). It included an albumin promoter to limit expression primarily to the hepatocytes. Although some factor VIII clotting activities suggested a possible effect that was independent of infused factor VIII required to treat sporadic routine bleeding episodes, there was no definitive, sustained response and levels found were near the level of detection so may have been spurious. The one study patient experienced a transient fall in his platelet count and showed transient laboratory evidence of liver cell damage, presumably as a toxic effect of the capsid or coating protein of the transducing vector system.

Table 2: Clinical Trials of Gene Transfer for Hemophilia A and B

Vector/promoter (study sponsor)	Delivery/primary organ	Subjects (N)	Results	Complications	Reference
Hemophilia A:					
Non-viral/fibronectin with B domain-deleted factor VIII cDNA (Transkaryotic therapies)	omental implantation after *ex vivo* electroporation, selection & growth of autologous, cultured fibroblasts	12	• no sustained responses • possible transient, VIII levels in some but at level of detection • possible decreased infusion requirements in some	no significant adverse events; requires skin biopsy and laparoscopic surgery with VIII	Roth et al, 2001
Retroviral/viral-LTR with B domain-deleted factor VIII cDNA (Chiron)	3 daily intravenous doses/liver	13	• no sustained responses • possible prolonged survival of transfused VIII in some • possible decreased infusion requirements in some	none observed	Powell et al, 2003
Adenoviral/albumin with full length factor VIII cDNA (GenStar)	intravenous/liver	1	• no sustained response • possible transient VIII levels but at level of detection	transient liver dysfunction and thrombocytopenia	White, 2003
Hemophilia B:					
Adeno-associated viral, AAV-2/CMV with factor IX cDNA and partial intron (Avigen)	intramuscular/multiple injections into vastus lateralis bilaterally	8	• no sustained responses • persistent vector & expression demonstrated up to 10 mo in muscle biopsies	no significant adverse events; IX given to prevent intramuscular bleeding	Manno et al, 2003
Adeno-associated viral, AAV-2, alpha-antitrypsin with factor IX cDNA (Avigen)	intra-hepatic via catheter with transient balloon occlusion of flow to hepatic artery	7	• no sustained responses • at highest dose, one patient achieved transient IX level of 12% • possible decreased infusion requirements in some	transient liver dysfunction in one with highest dose; IX given to prevent procedural bleeding	High, 2004; High et al, 2004

Hemophilia B

Two trials have been concluded using adeno-associated viral vector constructs in 15 subjects with hemophilia B (High, 2004). Although factor IX is generally expressed at higher levels than factor VIII, it is smaller than albumin and thus has a larger volume of distribution. Furthermore, on a molar basis, it circulates in a 300-fold excess over factor VIII. The first trial targeted gene transfer into muscle cells in severe hemophilia B subjects, and though long-term gene expression was demonstrated, systemic factor IX levels were not significantly increased. In the second trial, the liver was the target organ. At the highest dose, transient efficacy was demonstrated though one subject experienced delayed liver toxicity associated with this vector.

The "muscle" trial used an adeno-associated viral vector with a viral promoter expressing a factor IX "minigene" that contained the factor IX cDNA with a partial intron sequence (Manno et al, 2003). As doses escalated, multiple injection sites into the lateral thigh were performed. Muscle biopsies, performed at 2 and up to 10 months, showed persistent vector and, by immunofluorescence, factor IX protein expression. The only complications of note were related to the biopsy sites. Of note, no inhibitors developed; semen samples were screened and were negative for viral sequences excluding significant germline transmission. Results of the efficacy, however, were disappointing compared to the dose responses observed in preclinical studies including those in hemophilic dogs. This may relate to a relatively low density of cell surface receptors for adeno-associated virus in the muscle group selected for human studies, the serotype used, and the fact that myocytes and myotubes have a limited ability to γ-carboxylate factor IX. Thus more injection sites would be needed as opposed to larger amounts of vector injected per site.

In the "liver" trial, the adeno-associated viral vector was engineered for a higher level of expression and a hepatocellular-specific promoter was used (High, 2004). The vector was delivered through a catheter fluoroscopically placed into the hepatic artery as a radiologic procedure. A balloon was inflated to temporarily stop the hepatic circulation as used for localized infusion of chemotherapy for malignancies. The portal circulation prevented liver ischemia. Efficient hepatic gene transfer was achieved as one of two patients treated at the highest vector dose had factor IX clotting activities that reached 12%, clearly a significant increment due to the gene transfer. However, success was only temporary as activity fell to baseline by four weeks and at this time the patient showed transient laboratory evidence of liver cell toxicity. An additional patient, entered at a reduced vector dose, showed slight chemical liver dysfunction that may also reflect an immune response to the incoming vector capsid proteins (High et al, 2004). This complication led to the discontinuation of this trial (Kaiser, 2004) and ongoing research is focused on means to limit this potential toxicity.

Human Safety Considerations with Viral Vectors

Retroviral vectors

Retroviral vectors are packaged in producer cells by viral gene products that are supplied in "trans" by helper constructs. Precautions are needed to ensure that replication competent virus is not produced through recombination of helper and vector genes. Insertional mutagenesis is another safety concern because sites of retroviral vector integration cannot be controlled. Since the vector carries its own promoter that can activate any nearby genes, integration in proximity to a proto-oncogene could predispose that cell and its progeny to develop a neoplastic growth pattern, i.e., cancer. Considering the size of the entire genome, this is a very unlikely complication even if integration is not entirely random. There is considerable evidence in patients who have received older forms of retroviral vectors with a variety of different cDNAs that these retroviral vectors are safe and well tolerated, at least short term. However, the development of a T cell leukemia in two patients treated in a recent immunodeficiency gene therapy trial suggests that this type of complication can occur in certain, probably rare, instances, depending upon the type of gene being delivered, and the selection of cells for delivery, (Williams & Baum, 2003). Clearly non-viral vectors would be preferable to avoid any risk of replication competent virus generation or insertional mutagenesis. However, non-viral vectors are much less efficient at entering cells such that

delivery and stability must be improved considerably before they can be considered as potentially therapeutic.

Adenoviral vectors

Adenoviral vectors are safe in the sense that they do not integrate. Several copies can transduce the same cell and the copy number needs to be kept low enough to prevent cellular toxicity and cell death. As a cause of the common cold, it is possible that some individuals will have sufficiently high antibody titers against the parent type of adenoviral vector to prevent cell entry. Adenoviral vectors also activate the early, innate phase of the host immune system that, in its most extreme form, can result in an overwhelming systemic inflammatory state and death (Raper et al, 2003). To avoid the later, adaptive immune responses to adenoviral proteins, the mini-Ad (gutless/helper-dependent) vectors are particularly appealing although their ability to persist for sufficiently long periods to provide sustained clotting factor production remains to be demonstrated and they will require further modifications to allow repeated transductions. A recent study in dogs suggests that this may become possible (Brown et al, 2004). A variety of strategies to limit the immune responses to adenoviral vectors are being investigated (Schlagen et al, 2004).

Adeno-associated viral vectors

Adeno-associated viral vectors persist for extended periods of time in host cell nuclei but only a small proportion of the vector integrates into the genome. The small amount of the vector that integrates raises the same concerns of oncogene activation that apply with retroviral vectors. This could be problematic in hepatic gene therapy to an individual who carries hepatitis C as that virus is already causing increased hepatocyte turnover. Administration into muscles avoids the hepatitis C risk although this route is known to be more immunogenic. Although only one of the few hemophilia B dogs injected intramuscularly with canine factor IX cDNA-AAV vector developed a transient anti-factor IX antibody response (Herzog & Dobrzynski, 2004), the incidence could be higher in human patients. The original dog colony used has a single missense mutation with undetectable plasma factor IX antigen although the presence of a messenger RNA factor IX transcript suggests that traces of a factor IX protein may be synthesized, at least intracellularly and this may decrease the overall risk of an anti-factor IX antibody developing. Gross gene alterations and nonsense mutations are more commonly associated with alloimmunization in both hemophilia A and B. There was no evidence for alloimmunization in the eight previously transfused severe hemophilia B patients (all with missense genotypes) receiving AAV intramuscularly (Manno et al, 2003). The alloimmunization rate in hemophilia A following concentrate therapy is at least an order of magnitude greater than in hemophilia B. However, due to its larger size and intravascular distribution, factor VIII delivered by an intramuscular route would likely have limited access to the circulation. An initial concern with AAV liver delivery was the transient appearance of vector in human semen (High, 2004). The presence in sperm cells, however, has been essentially excluded. Furthermore, in a murine model, the AAV vector failed to transduce sperm cells, even when incubated directly with sperm at very high levels (Couto et al, 2004). Thus germline transmission, although theoretically possible for viral vectors, does not appear to occur.

In the one individual who achieved the first unequivocal response to AVV-factor IX delivered to the liver, there was evidence of a delayed immune response and transient hepatic toxicity (High et al, 2004). The degree to which this represents a serotype issue with the vector and/or a host response not observed in preclinical studies remains to be determined.

Patient participation

Selection of study subjects requires careful consideration, especially in a bleeding tendency that can usually be controlled by infusion therapy (Ragni, 2004). It is critical to ensure that potential subjects are counseled as to possible risks. Complications encountered in clinical trials to date need to be presented in an unbiased manner. Furthermore, the potential subject's motivation must be carefully evaluated for altruism to advance the science, understanding that there is little to no chance of personal gain, especially in phase I/II clinical trials. Protocols must ensure that potential subjects are not simply trying to please the

investigator, especially where s/he is also that individual's treating physician.

It has generally been agreed that initial clinical studies should involve consenting adult hemophilic patients. Whether or not HIV-positive individuals should be eligible is a debatable issue. If so, certain anti-retroviral medications may interfere with retroviral vectors. On the other hand there are a number of hemophilic patients who are long-term survivors of HIV infection, some of whom have remained healthy even without protease inhibitor therapy despite infection over 20 years ago. Any inclusion of HIV-positive adults should require that they are currently asymptomatic, have low viral loads, and reasonable CD4 lymphocyte counts. An additional concern is the potential for germline transduction. Although this is very unlikely for most of the vectors being proposed for clinical trials, it would seem prudent to select patients initially that will not be having children or are willing to use a barrier form of contraception for a period of time around the vector administration. Careful follow-up screening, such as with PCR amplification, to look for even traces of vector DNA in sperm samples, for example, should help settle the issue of whether or not germline integration is even a slight risk.

In the United States, the Food and Drug Administration (FDA) needs to review the preclinical data and approve each protocol for gene therapy before the vector can be considered as an investigational new drug (IND) and clinical experimentation can proceed. Furthermore, a national recombinant advisory board committee (RAC) of the National Institutes of Health (NIH) provides additional guidance and oversight for gene therapy protocols. Once the qualifications for inclusion in a study are determined and protocols are finalized and approved nationally, further consideration and approval of the protocols and consent forms is required by institutional review boards (IRBs) for human subjects of each participating institution. Rigorous safety standards are required to ensure safe, reproducible manufacture of the viral vectors. Even though there are perhaps slight and possibly unforeseen risks of gene therapy for hemophilia, animal model data suggests it can be effective and safe. Its safety to date in other patient groups strongly supports the careful design of clinical studies to determine the ability of gene therapy to lessen the severity of the bleeding tendency, and possibly to cure, factors IX or VIII deficiencies in patients with hemophilia B and A, respectively.

Concluding Comments

Hemophilia continues to represent a leading candidate condition for the successful application of gene therapy. Over the past decade, dramatic advances have been made in the preclinical assessment of hemophilia gene transfer and there are now many reports of long-term success in hemophilic mice and some success, lasting for several years, in hemophilic dogs. More recently, the initial human trials of hemophilia gene therapy have not shown any persistent adverse effects, but increments of clotting factor levels have been at best minimal and short lived. It seems likely that for long-term therapeutic levels of clotting factor to be achieved in humans, several remaining challenges will need to be overcome including the avoidance of host immune responses to the vector and the transgene product, increased transduction efficiencies, and the development of transgene constructs that maintain persistent expression following administration. However, despite these challenges, there is still reason for optimism, and the development of safe, effective methods to provide a "cure" for hemophilia remains feasible, hopefully within the next decade.

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