

GENE THERAPY IN HAEMOPHILIA

Ian Peake

Introduction

Haemophilia A (factor VIII {FVIII} deficiency) and haemophilia B (factor IX {FIX} deficiency) are rare X-linked bleeding disorders occurring at an incidence of 1:5,000 and 1:25,000 males throughout the world. Treatment of these conditions by replacement therapy with plasma-derived FVIII or FIX concentrates has been established in the developed world since the mid 1970s, but has brought with it the transmission of blood-borne viruses such as HIV and hepatitis B and C. Although today viral inactivation procedures have virtually eliminated such transmission, concerns over possible new viruses remain. Also in much of the world, blood transfusion services are unlikely to ever be able to meet the demand for blood product required to treat haemophilia effectively.

The identification and cloning of the FVIII and FIX genes and their expression in mammalian cells in culture some 10 years ago has resulted today in the availability of recombinant FVIII and FIX, although such products are expensive and have had little impact on the lack of treatment available to over 80% of the world's haemophiliacs. However, these studies have opened up the possibility of gene therapy for haemophilia and the dream of a "cure". For the reasons outlined below, many gene therapy studies have focused upon haemophilia B and FIX.

Gene therapy for haemophilia is achieved when the FVIII or FIX gene, once inserted into cells or tissues in the body of a haemophiliac, expresses the missing factor which is then secreted from the cell into the blood in quantities sufficient to correct the bleeding diathesis. However as a first approach it would also be worthwhile if plasma levels of the factor could be achieved such as to convert a severe case into a mild one by a modest 5–10% increase in plasma level.

In order to achieve gene therapy, the gene and appropriate expression sequences must be inserted into normal cells, either *ex vivo* followed by transplantation back into the body or *in vivo*. This is performed either by transfection (by cell membrane disruption) or transduction (controlled insertion) of the DNA in a vector. Vectors are usually viral based although non-viral systems are increasingly being studied. The properties of vectors are summarised in Table 1 and are discussed below.

Table 1. Gene therapy vectors.

Vector	Type/size Limit	Cell Division	Genomic Integration	Copy Number	Transfection Stability
Retroviral	RNA/<8kb	Yes	Yes	Low	High
Adenoviral	DNA/~8kb	No	No	High	Low
Non-viral	DNA/~50kb	No	No	High	Low

Vectors

Viral Vectors

Retroviral based vectors are most commonly used, based upon oncogenic animal viruses such as Moloney leukemia RNA virus. Such viruses are engineered to be non-infective by the removal of essential gag, pol and env encoding genes and are allowed to replicate in packaging cells containing defective helper viruses. Up to 8kb of RNA insert can be accommodated, and only dividing cells are transduced, thus restricting the occasions when such vectors are effective. However, transfection efficiency can be high in cells with a high cell surface viral receptor density, and the therapeutic gene is inserted into the cell's genome, albeit at random sites. This genomic integration results in stable transfection of the cell and its progeny, but a restriction on the number of copies integrated per cell is advisable in order to minimise the possibility of integration at a site in the genome, which could result in an altered cellular phenotype.

Adenovirus-based vectors have the advantage of being able to transduce non-dividing cells with high efficiency. Such vectors can accommodate up to 8kb of DNA insert and, since genomic integration does not generally occur, a high copy number per cell is allowed, limited only by the virus' ability to cause cell lysis. Because the therapeutic gene is not integrated but exists episomally within the nucleus, transfection stability is low. Also, re-exposure to adenoviral vectors is restricted by cellular immune responses resulting in immunity.

Although most viral based vectors have utilised retroviruses or adenoviruses, other viruses including adeno-associated viruses (parvovirus) have been studied. This virus can accommodate up to 5kb of DNA and transduce both dividing and non-dividing cells, and genomic integration can occur. However, vector rearrangement can also occur, and a second virus is required for replication. In the haemophilias, gene therapy studies utilising viral based vectors have been restricted to retro- or adenoviral systems

Non-Viral Vectors

Early methods of in vitro transfection of plasmid DNA involved calcium phosphate precipitation or procedures utilising various cations (polylysine, polybrene) and resulted in variable transfection efficiency, low rates of genomic integration and transient expression. They rely on non-specific endocytosis, and endosomal contents are invariably exposed to lysosomal enzyme destruction. Transfection efficiency can be improved by physical methods including electroporation. Improved techniques have used DNA particles complexed with cationic lipids. Ligand-mediated transfection has also been developed in which ligands (e.g., transferrin) to specific cell surface receptors are complexed to the DNA or the DNA-polylysine complex. This leads to receptor-specific endocytosis of the complex into the cell. In order to enhance endosomal release of the DNA and passage into the nucleus before lysosomal enzyme destruction, adenoviral particles can be added to the transfection mix. Endosomal lysis is enhanced by the presence of penton protein on the surface of the virus. This effect can be further increased by complexing the adenoviral particle to the DNA-ligand complex.

In vivo non-viral gene delivery can utilise many of the ideas outlined above, in particular to target specific cells. Thus asialoglycoproteins have been used as ligands to target liver cells, in complexes with polylysine-DNA plasmid-adenoviral condensates.

Direct administration of purified plasmid DNA is also possible by direct injection into muscle but, as might be expected, the efficiency of gene transfer and expression in such systems is relatively low.

Gene Therapy in Haemophilia B

The FIX cDNA (1.8kb) is small enough to be inserted in a range of retroviral and adenoviral vectors. Together with the stability of FIX and its ready measurement, this has led to many studies in which the FIX gene has been transduced into a variety of cells in culture (see review by Thompson⁽¹⁾). A range of cells including hepatocytes, endothelial cells, myoblasts, bone marrow stromal cells, keratinocytes and fibroblasts have been shown to effectively express normal human or canine FIX in culture and to express low levels in vivo after transplantation. However, levels in plasma have generally been below the therapeutic threshold, and even though genomic integration has occurred, the expression may be transient either due to cross-species antibody production or to epigenetic phenomena, which are poorly understood but which appear to switch off the DNA promoter sequences incorporated within the expression vectors.

However, these developments resulted in the first and to date only human gene therapy haemophilia experiment. Two brothers with mild haemophilia B were treated by gene therapy in China as reported by Lu and colleagues in 1993.⁽²⁾ Primary skin fibroblasts from each boy were cultured and transduced with a mixture of two retroviral vectors each containing the human FIX cDNA. In one vector the FIX gene was under the control of a cytomegalovirus (CMV) promoter; in the other, the retroviral LTR sequence assumed this control. Following successful transduction and a series of safety checks, the fibroblasts were mixed with rat tail collagen and transplanted subdermally into each boy. An initial dose of 10^7 cells was followed by several increasing doses over a period of 100 days. In the younger boy the FIX level rose from approximately 3% to 6% of normal (measured by procoagulant activity), but no rise was seen in the second child. However, FIX antigen appeared to increase in both. No follow-up data is available on this interesting study and these results have not been confirmed.

The in vitro studies outlined above showed that, because of the low level of FIX gene expression seen, larger numbers of cultured cells would need to be transplanted in order to achieve therapeutic plasma levels.

In vivo FIX gene transfer has been reported by Kay and colleagues^(3,4) using the haemophilic B dog model. A canine FIX cDNA retroviral vector was infused into partial hepatectomised haemophilic dogs via the hepatic portal vein.⁽³⁾ The partial hepatectomy was performed in order to induce hepatitic cell division and so enable retroviral transduction to occur. One percent transduction was seen and a slight increase in plasma FIX level (0.1%) was sustained over several months. Perhaps more significantly, the WBCT of the treated dogs reduced from 50 to 20 mins (normal up to 10 mins). Although clearly the efficiency of both vector transduction and expression were poor, the experiment was of limited success.

A similar experiment was then reported using a canine FIX cDNA adenoviral vector.⁽⁴⁾ A 25% hepatocyte transduction rate was observed, and a rise in plasma FIX to 300% of normal was quickly observed. However, as expected, this was transient and

levels fell to below 1% in 30 days. Importantly, a second administration of the vector was completely unsuccessful due to the immune response elicited by the adenoviral antigens in first experiment.

More recent studies have addressed some of these problems. Dai and colleagues⁽⁵⁾ have shown that the immune response in mice both to the adenoviral antigens and to canine FIX produced after *in vivo* transduction can be reduced by the use of immunosuppressive drugs (cyclosporin A and cyclophosphamide) and that tolerance can be induced. Cyclosporin A has also been shown to prolong FIX secretion in haemophilia B dogs after adenoviral based transduction.⁽⁶⁾

Finally, progress has been reported utilising non-viral based vectors. Cristiano and colleagues⁽⁷⁾ showed that FIX cDNA-polylysine-asialoglycoprotein conjugates could effectively transfect cells *in vitro* and that this was enhanced by the presence of adenoviral particles (see above). Percales et al⁽⁸⁾ have used a chimeric gene containing the phosphoenolpyruvate carboxykinase (PEPCK) gene promoter linked to the human FIX gene cDNA, which was condensed with galactosylated polylysine. The efficiency of transduction of liver cells *in vivo* in the rat as assessed by plasma FIX levels was related to the structure of the complex, i.e., the level of condensation. Also the level of transcription of the FIX gene could be regulated, with significant induction on a high protein, low carbohydrate diet. Liposomal-encapsulated FIX cDNA controlled by a retroviral LTR sequence is another approach to *in vivo* transduction.⁽⁹⁾

Gene Therapy in Haemophilia A

Although gene therapy for haemophilia A would be medically of more significance than for haemophilia B, the size of the FVIII cDNA (8.8 kb) means that it cannot be successfully inserted into any known viral based vector. Studies have therefore used a truncated FVIII cDNA in which the region encoding the central B domain of FVIII has been deleted (D B FVIII cDNA). D B FVIII appears to function in an identical manner to full length FVIII in blood coagulation and has been recently shown to be clinically effective in clinical trials of recombinant D B FVIII in patients with haemophilia A.⁽¹⁰⁾

Initial *ex vivo* and *in vivo* experiments using fibroblasts and bone marrow cells indicated that D B FVIII cDNA within a retroviral vector could be expressed at a low level *in vitro* but generally not *in vivo*.^(11,12) The only report of significant levels of FVIII expressed *in vitro* and *in vivo* by Dwarki et al⁽¹³⁾ used the MFG retroviral vector system and resulted in highly efficient transduction. These studies have yet to be confirmed. Connelly and colleagues⁽¹⁴⁾ have reported encouraging results with an adenovirus based D B FVIII cDNA vector. D B FVIII at therapeutic levels was observed *in vivo* after tail vein injection, and expression continued *in vivo* for several weeks. These studies are encouraging and indicate that the transient expression *in vivo* seen with other adenoviral based systems (see above) as a result of rapid vector elimination and/or immunity to the vector may be related to the vector itself and the dose used.

Zatloukal and colleagues⁽¹⁵⁾ have shown high level expression of D B FVIII in primary mouse fibroblasts and myoblasts using a receptor-mediated, adenoviral-augmented gene delivery system. Transduction of cells was achieved by complexing the

human D B FVIII cDNA–CMV promoter construct to defective adenoviral particles and to transferrin-polylysine. Results showed over 30% transfection efficiency with levels in vitro of 1 unit of FVIII per 10^6 cells per 24 hrs. Therapeutic plasma levels of FVIII were also obtained when the transfected fibroblasts were injected into the spleen or liver in vivo, but not when myoblasts were implanted into muscle. This may be related to the size of the FVIII molecule and its inability to diffuse out of the muscle.

Although encouraging, levels of FVIII expressed after transduction or transfection are low compared to FIX or to the homologous protein, factor V. This is particularly true for retrovirus based vectors and appears to relate to the FVIII gene itself. Koeberl et al⁽¹⁶⁾ have identified a 1.2 kb inhibitory sequence within the FVIII cDNA which decreases steady state RNA levels from a retroviral vector by 30- to 100-fold. Marquette and colleagues⁽¹⁷⁾ have identified a 110 amino acid region within the A1-domain of FVIII which inhibits secretion of FVIII from mammalian cells due to intracellular binding of the protein to the protein caperonin BiP within the endoplasmic reticulum. This sequence is not found in factor V. These inherent blocks to effective FVIII expression are being studied in several laboratories. Changes to the 1.2 kb inhibitory sequence have, to date, failed to increase expression.⁽¹⁸⁾ However, the same authors have shown that the insertion of an intron 5' to the FVIII cDNA within a retroviral vector results in a 20–40-fold increase in expression.

Future Prospects in Haemophilia **Gene Therapy**

Low levels of expression and instability of the transferred gene are the major problems related to gene therapy in haemophilia. Advances in vector design are essential to overcome these hurdles. Retroviral vectors with increased levels of expression resulting from better promoters, probably tissue specific, are required and such a vector capable of transducing resting cells would be a significant advance. The AIDS virus, HIV, has this latter property and could be used as a model. Adenoviral vectors could be developed to give persistent expression and a reduced immune response. Other viruses are being examined which may have some of the above properties, and any virus that results in significant levels of genomic integration will be beneficial. Non-viral based vectors have significant advantages in terms of insert size (the complete FVIII cDNA could be inserted) and with regard to cell or tissue targeting. They are also inherently safer than virus based systems. Much effort is therefore being addressed to make them more efficient by increasing endocytosis and reducing lysosomal degradation. Increased intra-nuclear stability and possible genomic integration by including those sequences from retroviruses which perform these functions are other approaches being studied.^(19,20)

As indicated above, the inherent properties of the FVIII gene significantly affect its own expression. Alterations to the gene which will not affect the properties of the expressed protein are being examined, and the introduction of an intron sequence into the vector has already increased levels of expression.⁽¹⁸⁾ It may be that similar studies will result in increased expression of the FIX gene.

At present, gene therapy for haemophilia appears to be something for the future. Significant advances need to be made as outlined above. Perhaps transient cure using

transiently expressed adenoviral or non-viral vectors will be the first benefit from these studies.

References

1. Thompson AR. Progress towards gene therapy for the haemophilias. *Thrombosis and Haemostasis* 74: 45.1995
2. Lu D-R, Zhou J-M, Zheng B, Qui XF, Xue JL, Wang JM, Meng PL, Han FL, Ming BH, Wang XP. Stage 1 clinical trial of gene therapy for haemophilia B. *Sci China (B series)* 36: 1342. 1993
3. Kay MA, Rothenberg S, Landen CN, Bellinger DA, Leland F, Toman C, Finegold M, Thompson AR, Read MS, Brinkhous KM, Woo SLC. In vivo gene therapy for haemophilia B: Sustained partial correction in factor IX deficient dogs. *Science* 262: 117. 1993
4. Kay MA, Landen CN, Rothenberg SR, Taylor LA, Leland F, Wiehle S, Fang B, Bellinger D, Finegold M, Thompson AR, Read M, Brinkhous KM, Woo SLC. In vivo hepatic gene therapy: Complete albeit transient correction of factor IX deficiency in haemophilia B dogs. *Proc Natl Acad Sci USA* 91: 2353. 1994
5. Dai Y, Schwarz EM, Gu D, Zhang W-W, Sarvetnick N, Verma IM. Cellular and humoral immune responses to adenoviral vectors containing factor IX gene: Tolerization of factor IX and vector antigens allows for long-term expression. *Proc Natl Acad Sci USA* 92: 1401. 1995
6. Eisensmith RC, Fang B, Kay MA, Landen CN, Cross RE, Bellinger DA, Read MS, Hu PC, Brinkhous KM, Woo SLC. Gene therapy for hemophilia B: Cyclosporin treatment increases the persistence of adenovirus-mediated factor IX expression in hemophilia B dogs. *Blood* 84: 225a. 1994
7. Cristiano RJ, Smith LC, Kay MA, Brinkley BR, Woo SLC. Hepatic gene therapy: Efficient gene delivery and expression in primary hepatocytes utilizing a conjugated adenovirus-DNA complex. *Proc Natl Acad Sci USA* 90: 11548. 1993
8. Perales JC, Ferkol T, Beegen H, Ratnoff OD, Hanson RW. Gene transfer in vivo. Sustained expression and regulation of genes introduced into the liver by receptor-targeted uptake. *Proc Natl Acad Sci USA* 91: 4086. 1994
9. Baru M, Axelrod JH, Nur I. Liposome-encapsulated DNA-mediated gene transfer and synthesis of human factor IX in mice. *Gene* 161: 143. 1995
10. Fijnvandraat K, Berntorp E, Ten Cate JW. Recombinant B domain deleted FVIII (rVIII-SQ): pharmacokinetics and initial safety aspects in hemophilia A patients. *Blood* 1996 in press.
11. Hoeben RC, van der Jagt RCM, Schoute F, van Tilburg NH, Verbeet M Ph, Briët E, van Ormondt H, van der Eb AJ. Expression of functional factor VIII in primary human skin fibroblasts after retrovirus-mediated gene transfer. *J Biol Chem* 265: 7318. 1990
12. Hoeben RC, Einerhand MPW, Briët E, van Ormondt H, Valerio D, van der Eb AJ. Towards gene therapy in haemophilia A. Retrovirus-mediated transfer of a factor VIII gene into murine haematopoietic progenitor cells. *Thrombosis and Haemostasis* 77: 341. 1992

13. Dwarki VJ, Belloni P, Nijjar T, Smith J, Couto L, Mireille R, Clift S, Berns A, Cohen LK. Gene therapy for hemophilia A: Production of therapeutic levels of human factor VIII in vivo in mice. *Proc Natl Acad Sci USA* 92: 1023. 1995
14. Connelly S, Smith TAG, Dhir G, Gardner JM, Mehaffey MG, Zaret KS, McClelland A, Kaleko M. In vivo gene delivery and expression of physiological levels of functional human factor VIII in mice. *Human Gene Therapy* 6: 185. 1995
15. Zatloukal K, Cotten M, Berger M, Schmidt W, Wagner E, Birnstiel ML. In vivo production of human factor VIII in mice after intrasplenic implantation of primary fibroblasts transfected by receptor-mediated, adenovirus-augmented gene delivery. *Proc Natl Acad Sci USA* 91: 5148. 1994.
16. Koerberl DD, Halbert CL, Krumm H, Miller AD. Sequences within the coding regions of clotting factor VIII and CFTR block transcriptional elongation. *Human Gene Therapy* 6: 469. 1995.
17. Marquette KA, Pittman DD, Kaufman RJ. A 110-amino acid region within the A1-domain of coagulation factor VIII inhibits secretion from mammalian cells. *J Biol Chem* 270: 10297. 1995.
18. Chuah MKL, Vandendriessche T, Morgan RA. Development and analysis of retroviral vectors expressing human factor VIII as a potential gene therapy for haemophilia A. *Human Gene Therapy* 6: 1363. 1995
19. Cristinao RJ, Roth JA. Molecular conjugates: a targeted gene delivery vector for molecular medicine. *J Mol Med* 73: 479. 1995.
20. Ledley FD. Non viral gene therapy: The promise of genes as pharmaceutical products. *Human Gene Therapy* 6: 1129. 1995.