

Gene therapy for haemophilia

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Summary

Gene therapy is an appealing prospect for the treatment of human diseases. In this chapter, I will describe the hopes that gene therapy has brought for hemophilia patients, as well as the hurdles that the researchers have encountered on the route that shall lead to the development of a clinically applicable protocol.

I. Introduction

Haemophilia is a congenital coagulation disorder characterized by uncontrolled haemorrhagic episodes that are crippling and potentially life-threatening. Haemophilia A results from subnormal levels of an essential cofactor protein, factor VIII (F.VIII), and affects 1 in every 10,000 males; haemophilia B is associated with a lack of an essential protease, factor IX (F.IX), and occurs in 1 out of 50,000 males. Due to the absence of these key intermediates in the clotting cascade, haemorrhage is the most frequent cause of death in untreated haemophiliacs.

To date protein-replacement therapy is the treatment of choice. This treatment essentially normalized both the life expectancy and the quality-of-life. Notwithstanding its tremendous achievements, this therapy has several drawbacks. The treated patient is still prone to spontaneous haemorrhages with the associated risk of chronic joint damage. In addition, therapy with plasma-derived F.VIII has resulted in transmission of several human viruses, such as HIV and hepatitis viruses. The risk of exposure to blood-borne pathogens has been virtually eliminated by improved manufacturing procedures and, more recently, by application of recombinant-DNA-derived F.VIII. Nevertheless, the ideal therapy would be independent of blood-derived products (Peake et al., 1993) and would provide a sustained therapeutic effect. Gene therapy may hold the promise of such a treatment of haemophilia and could, in theory, completely cure the disease.

II. Basic strategies for hemophilia gene therapy

Two strategies are being pursued for haemophilia gene therapy. In the *ex-vivo* gene-transfer approach, cells are isolated from the patient, cultured and genetically modified in the laboratory. The treated cells, that now should synthesize the factor VIII protein, are reimplanted into the patient in order to bring about a continued production of the desired clotting factor. For this approach, skin fibroblasts, keratinocytes, endothelial cells, hepatocytes, hematopoietic progenitor cells, and myoblasts have been considered. Alternatively, the *in-vivo* approach aims at genetic modification of some of the patients cells *in-situ*. In this strategy, gene-transfer vehicles are administered to the patient and should deliver the genes to the tissue of interest. This approach concentrates on genetic modification of the liver, the main site of factor VIII synthesis in healthy individuals (see Fallaux and Hoeben (1996) for a more extensive review).

III. Gene Therapy for Hemophilia A

A. Relevant properties of factor VIII

The F.VIII protein is a large multimeric glycoprotein (300 kDa) that circulates in plasma in low concentrations. The protein is synthesized mainly in the liver as a single-chain polypeptide, which by intracellular processing, is converted in a two-chain dimer of 80-kDa and 200-kDa subunits. Before the actual activation of the F.VIII protein, a large segment of the 200 kDa subunit (the B-domain), is

removed, resulting in a 90-kDa heavy chain complexed to the 80-kDa light chain. Further proteolytic cleavage activates the F.VIII protein (Pittman and Kaufman, 1989; Pittman et al., 1994). The F.VIII protein is translated from an mRNA of approx. 9000 nt, of which 7053 nt are coding. The F.VIII gene, located on the X-chromosome, is about 186.000 bp in size.

Production of recombinant DNA-derived F.VIII using the human F.VIII cDNA has been difficult. Firstly, the F.VIII cDNA has been found to contain sequences that repress its expression, resulting in low levels of F.VIII-specific mRNA (Lynch et al., 1993; Hoeben et al., 1995). Secondly, the majority of the F.VIII protein is transported inefficiently from the endoplasmic reticulum to the Golgi system due to retention of the protein in the ER (Pittman and Kaufman, 1989; Pittman et al., 1994). Thirdly, the protein is extremely sensitive to proteolytic degradation and needs to be stabilized by the von Willebrand factor. In addition, the protein undergoes extensive post-translational modification and needs to be proteolytically cleaved for its functional activation (Pittman and Kaufman, 1989).

B. Ex-vivo gene therapy: problems with retroviral vectors.

Many studies focused on the development of retroviral vectors for transfer of a F.VIII gene. In all studies published so far, F.VIII cDNA clones were used in which the non-essential B-domain was removed. The sequences coding for the 90-kDa heavy chain were fused in-frame to the 80-kDa light chain codons. Removal of the B-domain does not significantly affect any known function of the protein; the complete and the B-domain-deleted F.VIII variants are virtually identical in functional assays (Pittman et al., 1993). These B-domain-deleted cDNA clones have a size of approx. 4,500 base pairs, and therefore can be inserted in retroviral vectors without exceeding the packaging capacity of the virus. Retrovirus-mediated transfer of the B-domain-deleted F.VIII cDNA has been achieved into various cell types, e.g., skin fibroblasts (Israel and Kaufman, 1990; Hoeben et al., 1990; Lynch et al., 1993), endothelial cells (Chuah et al., 1995; Dwarki et al., 1995), myoblasts (Zatloukal et al., 1994), and haematopoietic progenitor cells (Hoeben et al., 1992). The F.VIII secreted by these cells was functional, illustrating that also cells of non-hepatic origin have the capacity for proper post-translational modification of the F.VIII protein. This illustrates the idea that gene therapy for haemophilia is not necessarily restricted to genetic modification of the hepatic cells that normally produce F.VIII.

In general, synthesis of F.VIII by genetically modified cells in culture has been quite low. Both the titre of the retroviral vectors, and the amounts of F.VIII secreted by the transduced cells are reduced about 100-fold in

comparison to FIX and other cDNAs (Lynch et al., 1993; Hoeben et al., 1995). The low titres and the reduced amounts of F.VIII produced are caused, at least in part, by the very low amounts of F.VIII-specific transcripts that accumulate in the transduced cells (Lynch et al., 1993). There is now ample evidence that the inhibition of expression is caused by sequences in the F.VIII cDNA itself and that repression occurs at the level of transcription (Lynch et al., 1993; Hoeben et al., 1995; Koeberl et al., 1995). Lynch et al. (1993) located a 1.2-kb stretch of the F.VIII cDNA ('INS') that reduces the titre of the F.VIII retroviral vectors. These sequences inhibit the F.VIII mRNA accumulation in the cytoplasm. In independent experiments we identified a 305-bp region in the F.VIII cDNA that is involved in the repression phenomenon (Hoeben et al., 1995). Intriguingly, the latter fragment is located near the 'INS' region. In the 305-bp region, sequences were found that resemble the Autonomously Replicating Sequence-consensus (ARSc) sequences of yeast, and the A/T rich sequences found in mammalian Matrix-Attachment Regions (MAR). It has been shown that multimerization of the F.VIII cDNA-derived ARSc/MAR-like sequences could functionally mimic the repression phenomenon when linked to a heterologous reporter gene. Also, de-repression of expression by sodium butyrate could be mimicked using multimers of the F.VIII-derived sequences. This suggests that such ARSc/MAR-like sequences, dispersed throughout the F.VIII cDNA, may alter the chromosomal context of the F.VIII-expression vector (e.g. by associating to the nuclear matrix), resulting in repression of expression (Fallaux et al., 1996). In the F.VIII cDNA the presence of a number of multiple elements in the F.VIII cDNA could form a functional MAR. Such model can explain the difficulties in pinpointing the sequences involved in the repression. So far there is no evidence to support any physiological relevance for the presence of the repressor sequences in the F.VIII cDNA.

To improve the expression, Chuah and co-workers (1995) used a conservative mutagenesis strategy to introduce the maximum number of nucleotide changes in the 1200-bp 'INS' region. Despite their impressive efforts, this neither increased the virus titre nor F.VIII expression. However, the insertion of an intron in their retroviral vector increased F.VIII expression up to 20-fold, and boosted virus titres up to 40-fold. This correlated with an increase in mRNA accumulation, which suggests that the inclusion of an intron in the retroviral backbone relieved the transcriptional repression (Chuah et al., 1995).

Although the problematic expression has been found to occur with many retroviral vectors, some appear to be less prone to the inhibition. Dwarki and colleagues (1995) reported F.VIII expression levels and vector titres that are at least 10- to 100-fold higher than those reported by others. In this vector, based on the MFG retroviral vector, the F.VIII cDNA is located at the exact position of the

retrovirus *env* gene. Thus, the F.VIII message is translated from the spliced sub-genomic mRNA. Although its efficiency is not easily understood considering the repression that has been reported by others, it is the first F.VIII vector that meets the requirements with respect to efficiency of a clinically applicable retroviral vector.

C. Implantation of retrovirally-transduced cells

Several cell types can be considered as targets for genetic modification in a protocol for gene therapy for haemophilia. Diploid skin fibroblasts are attractive targets. These cells can easily be harvested from patients, can be grown to large numbers in tissue culture and can be transduced with retroviral vectors with relative ease. In initial experiments F.VIII-secreting fibroblasts of murine or human origin, embedded in an artificial collagen matrix, were implanted subcutaneously on the midbacks of nude mice. In the case of human fibroblasts, cells isolated from the grafts 8 weeks after implantation still had the capacity to secrete F.VIII when regrown in culture. These results demonstrate the persistence of the transplanted cells in a metabolically active state (Hoeben et al., 1993). Unfortunately, no human F.VIII could be detected in the recipients' plasma that might have been secreted by the implanted cells. This was attributed to the short half-life of the human F.VIII protein in mice. Dwarki and colleagues (1995) observed circulating F.VIII after intravenous and intra-peritoneal injection of recombinant F.VIII protein. In parallel experiments these authors could not detect human F.VIII following intra-muscular or subcutaneous injection. This can be due to the susceptibility of the protein to proteolysis, resulting in degradation of F.VIII before it can reach the circulation. After intra-peritoneal implantation of F.VIII-secreting fibroblasts into immunodeficient mice circulating human F.VIII could be detected (maximally 100 ng/ml) in their plasma for up to 10 days (Dwarki et al., 1995). The capacity of transduced cells to deliver the F.VIII into the circulation was dependent on the site of implantation. These data convincingly demonstrate the feasibility of this approach, although the persistence of expression obviously needs to be increased.

D. In-vivo gene therapy: encouraging results with adenoviral vectors.

Conceptually protocols involving *in-vivo* gene transfer are more straight forward than the *ex-vivo* approaches. Connelly *et al.* (1995) studied this approach using a recombinant adenoviral vector, Av1ALH81, in which the F.VIII cDNA is driven by a liver-specific mouse albumin promoter. The use of this vector circumvented many of the problems associated with retroviral vectors in *ex-vivo* gene transfer strategies. HepG2 hepatoma cells transduced with

Av1ALH81 secreted high levels of biologically active human F.VIII (>240 ng/10⁶ cells/24h). Administration of Av1ALH81 to mice resulted in an efficient transduction of the liver (the systemically administered adenovirus exhibits a strong hepatotropism). The resulting F.VIII levels in the recipients plasma peaked at 300 ng/ml. These levels are even more impressive if one considers the short half-life of the human protein in mice. Normal F.VIII levels in humans are 100-200 ng/ml, and levels as low as 10 ng/ml are therapeutic. Thus, the mice were producing human F.VIII at levels that exceeded those in normal human plasma. In the recipient mice F.VIII levels in plasma peaked at day 7, and decreased slowly to background levels 7 weeks after treatment. The decline in plasma F.VIII levels correlated with the loss of vector DNA from the liver. This is caused by elimination of the transduced hepatocytes by the hosts' immune system (Yang et al., 1994; Engelhardt et al., 1994). An optimized F.VIII adenoviral vector, Av1ALPH81, was generated that carries an intron in the F.VIII expression cassette (Connelly et al., 1996b). The F.VIII plasma levels (up to 2.000 ng/ml) in mice that received this vector exceeded those obtained with Av1ALH81. This allowed the administration of lower, less toxic vector doses while maintaining sufficient levels of human F.VIII in the plasma of the recipient mice. F.VIII levels in plasma in the therapeutic range persisted for at least 22 weeks after a single administration of the vector (Connelly et al., 1996a) in mice. In hemophilic dogs the bleeding tendency could be completely, although transiently, corrected (Connelly et al., 1996c). This provided the much awaited proof-of-concept of gene therapy for hemophilia A in a large animal model for hemophilia A. It remains to be established whether also in the large animal models for haemophilia A (e.g. haemophilic dogs) and, ultimately, in humans, vector virus-doses can be found that combine adequate and persistent F.VIII levels in plasma with the absence of apparent hepatotoxicity.

IV. Gene therapy for haemophilia B

A. Relevant properties of Factor IX

The F.IX protein is much smaller in size (55 kDa), and 500 times more abundant on weight basis than F.VIII. Its gene is located on the X chromosome and is 33.000 bp in size. Whereas the F.VIII has no intrinsic enzymatic activity, the activated F.IX functions as a serine protease. It is secreted as an inactive precursor protein that can be activated by proteolytic cleavage. The F.IX protein is modified extensively. The first 12 glutamic-acid residues of the Gla domain are gamma-carboxylated post-translationally. This modification is essential for Ca²⁺ binding and F.IX function (reviewed by Roberts (1993)).

B. Status of hemophilia-B gene therapy

The developments in the field of gene therapy for haemophilia B paralleled, and often preceded, those for haemophilia A. Starting in 1987 (Anson et al., 1987), a variety of cultured cells have been transduced with retroviral F.IX vectors (reviewed by Fallaux and Hoeben, 1996). In general, functional F.IX was found to be secreted in significant amounts. However, transplantation of the transduced fibroblasts into mice, resulted in transient F.IX plasma levels that were lower than would be expected on the bases of the F.IX secretion *in-vitro* (Scharfmann et al., 1991; Axelrod et al., 1990; Palmer et al., 1989; Palmer et al., 1991; St.Louis and Verma, 1988). In some of the recipients the formation of F.IX inhibitors could be established, explaining the disappearance of circulating F.IX (St.Louis and Verma, 1988). In addition, the retroviral LTR-promoter that drives expression of the gene of interest was found to be inactivated in fibroblasts *in vivo* (Axelrod et al., 1990; Palmer et al., 1989; Palmer et al., 1991). Although the latter problem can be overcome by using a cellular promoter (St.Louis and Verma, 1988), such promoters are generally not very strong. Despite these problems, in 1993, Lu and colleagues initiated a phase-I gene-therapy trial with retrovirus-transduced autologous skin fibroblasts (Lu et al., 1993). Two brothers with haemophilia B were treated. It has been reported that in one patient F.IX-clotting activity increased significantly (from 2.9% to 6.3%), and persistently (over 6 months), but not in the other individual. Although encouraging, this trial is still a matter of debate (Thompson, 1995).

In parallel, many other cell types have been efficiently transduced with F.IX retroviral vectors, including myoblasts (Hortelano et al., 1996; Yao and Kurachi, 1992; Dai et al., 1992; Yao et al., 1994; Baru et al., 1995; Wang et al., 1996), endothelial cells (Axelrod et al., 1990; Yao et al., 1991), hepatocytes (Kay et al., 1993; Kay et al., 1994), keratinocytes (Gerrard et al., 1993; Gerrard et al., 1996; Fenjves et al., 1996), and haematopoietic cells (Hao et al., 1995). Although in laboratory animals circulating F.IX protein has been detected after transplantation of the genetically modified cells, in many cases the synthesis is low and transient, similar to the fibroblast-transplantation experiments. However, it can be anticipated that improvement in the vector technology and transplantation procedures may increase the F.IX levels considerably. Recently, also vectors derived from the adeno-associated virus have been used for the expression of F.IX in cultured cells (Chen et al., 1997) and *in vivo* (Koeberl et al., 1997). With these vectors significant levels of F.IX protein could be observed in the recipient mice up to 5 months post-infection. Although the expression is still low, the AAV-derived vectors capacity to infect non-mitotic cells makes it an important alternative for the retroviral vectors, especially for *in-vivo* liver-directed gene transfer.

The efficacy of *in-vivo* gene therapy for haemophilia has been demonstrated by Kay and collaborators (Kay et al., 1993). They infused F.IX retroviruses in haemophilic

dogs (Beagles) that had previously undergone partial hepatectomy to stimulate the remaining hepatocytes to divide. Despite the low amounts of F.IX produced (ca. 0.1 % of normal), the average clotting-time was reduced by approximately 60%. The production of the clotting factor persisted for over 9 months (Kay et al., 1993). These results are very promising, although a further 10-100 fold increase in production is required to reach a clinically beneficial range.

Also adenoviral vectors have been used for the gene transfer of a human F.IX gene into mice. After a single intra-venous dose into the tail vein, amounts of 400 ng/ml human F.IX could be detected in the recipient mice (Smith et al., 1993). However, the levels slowly decreased to baseline within the course of 10 weeks. A second administration of the virus did not re-establish human F.IX plasma levels. This was due to high amounts of circulating antibodies that were generated and neutralized the vector viruses upon re-challenge (Smith et al., 1993). Similar results have been obtained in F.IX-deficient dogs (Kay et al., 1994). After a single dose of the virus (administered into the portal vein) the bleeding tendency of these dogs was transiently corrected with an increase in F.IX levels from 0 to 300% of the level present in normal dogs. Although therapeutic levels could be maintained for 1-2 months, the F.IX levels decreased significantly in time.

To prolong the expression of the transduced F.IX gene, the administration of the adenovirus vector was combined with immuno-suppression by cyclosporin A, which allowed expression to persist up to 6 months (Fang et al., 1995). However, neutralizing antibodies were formed, making subsequent administrations of the vector ineffective. The occurrence of neutralizing antibodies could be reduced by transient immuno-suppression with deoxyspergualin or cyclophosphamide, allowing repeated administrations of the vector (Dai et al., 1995; Smith et al., 1996). It has been also been reported that, in mice, tolerance could be induced if the adenovirus was administered neonatally (Walter et al., 1996), allowing repeated administrations of the vector. However, given the differences in the development of their respective immune systems, this procedure can not be translated directly to dogs or humans.

In order to prolong the expression of F.IX without the need of immune suppression, vectors have been generated and tested in which the adenovirus E2A gene carries the *ts125* mutation which makes the protein product of the E2A gene, the single-stranded DNA Binding Protein (DBP), temperature sensitive. At the body temperature of mice and dogs, the *ts125* DBP is non-functional, resulting in a reduced level of adenovirus late-gene expression, and consequently, in reduced immuno-genicity. However, the *ts125* did not increase the persistence of expression neither in mice, nor in haemophilic dogs (Fang et al., 1996). An approach that appears more successful is to maintain the E3 region in the adenoviral vector. The protein products of

the E3 region can suppress host immune reactions by interference with the expression of MHC class I molecules and by other mechanisms. Side-by-side comparison of $\Delta E1/\Delta E3$ F.IX adenoviral vectors with $\Delta E1$ F.IX adenoviral vectors demonstrated a longer persistence of the expression with the former type (Poller et al., 1996). This strongly argues for use of vectors that have a wild-type E3 region. However, deletion of the E3 is often required to generate the space required for the insertion of the gene of interest, especially with larger genes (e.g. the F.VIII cDNA).

V. The future

Some of the hurdles on the road to gene therapy for haemophilia have been taken. The results obtained so far have demonstrated the potential efficacy and provided the conceptual 'proof-of-principle'. However, several aspects need to be improved before clinical application can be considered for the treatment of haemophilia. In the *ex-vivo* approaches the techniques for cell isolation, gene transfer and cell transplantation need further improvement. Also the persistence of expression and the level of expression need to be enhanced. On the *in-vivo* route it will be essential to efficiently target the gene-transfer vector to the desired tissue to ensure specific delivery of the curative gene into the cell type of choice. Ways must be found around the immune problems that restrict the applicability *in vivo* of the current adenovirus vectors. It will be essential to limit the cellular immune response directed against the transduced cells. Also the rapid humoral response which generates neutralizing antibodies that inhibit subsequent virus-mediated gene transfer, reduces the applicability. Although the results obtained with transient immuno-suppression of the recipients are promising, strategies in which the immunogenicity of the vector is reduced by removing all the viral protein-coding regions are preferable (Kochanek et al., 1996; Haecker et al., 1996; Chen et al., 1997). We should not forget that viruses although harmless in normal individuals, may become pathogenic in severely immune-compromised hosts. Even the C-group adenoviruses that we use as vectors, may become pathogenic if the immune system is compromised, e.g. after a bone-marrow transplantation (Hierholzer, 1992; Landry et al., 1987; Bertheau et al., 1996). Thus, we should adapt the vector to the patient, and not vice versa.

These issues above are not unique for haemophilia, but are imperative for all gene-therapy approaches for the treatment of congenital disorders. A concern that is more prominent in the case of haemophilia than in other disorders, is the potential humoral response against the transgene product (viz. F.VIII or F.IX). Such inhibitors, that also are formed in a minority of patients upon regular treatment, inhibit not only the genetic therapy but also the conventional replacement therapy. It needs to be

established at what frequency inhibitors (F.VIII or F.IX-antibodies) occur after the gene therapy. To determine such frequencies, studies must employ the homologous cDNA. The cloning of the canine F.IX cDNA (Evans et al., 1989b) and the murine F.VIII cDNA (Elder et al., 1993) permits to evaluate the gene-therapy procedures in the established canine (Mauser et al., 1996; Evans et al., 1989) and murine (Bi et al., 1995) models for haemophilia. This will allow a detailed comparison of the current and the future methods for haemophilia management with respect to safety and efficacy.

Notwithstanding the promising results, we should realize that gene therapy has only recently emerged as an approach for the treatment of various diseases. With the input from academic institutions and (biotech)-industry steadily growing, the number of potential applications, too, is increasing. Applications are found for the treatment of e.g. AIDS, cancer, arthritis, Parkinson's disease and many hereditary diseases. Some of these applications have already reached the stage of phase-I clinical trials.

With the increased input also the range of available tools is expanding. New viral-vector systems are being developed with improved applicability, yield and safety features. In addition, novel very efficient non-viral gene-transfer methods have been described that eventually may match and even surpass the efficiency of the viral vector systems. In this respect it is worthwhile to note how the viral and non-viral systems converge. On one hand the safety of viral gene-transfer systems is further increased by reducing the content of virus (-derived) products in the vector. On the other hand the non-viral vectors mimic the viral functions as much as possible using synthetic ingredients, resulting in artificial 'viroid-particles'. In this respect the pioneering work of Birnstiel and colleagues (Zatloukal et al., 1994), and others (Lozier et al., 1994; Ferkol et al., 1993) is exemplary and has already been used for the expression of clotting-F.VIII and IX in rodents. It is, therefore, reasonable to anticipate that the future will hold promise of vector systems that can be administered systemically and that will target the gene-of-choice to a predetermined target tissue in a very efficient and highly specific manner.

In addition to these "scientific" aspects we will need considerable efforts at the level of the production of the vectors. The type of therapeutics that is being considered for clinical application differs in several aspects from the more "conventional" drugs. Hence at the production side, considerable investments need to be made in order to acquire the technology to produce 'clinical-grade' vectors in sufficient quantities.

Gene-therapy research thus requires the concerted action of scientists from many disciplines, e.g. from fundamental research in virology, genetics and process technology to (pre-)clinical research in the fields of haematology, pediatrics and surgery. Once we have been

able to solve the 'scientific' and the 'technical' problems and only if we have unequivocally demonstrated the long-term safety and efficacy of this new technology, gene therapy can become a significant alternative for the current treatment of haemophilia.

Acknowledgments

I thank the members of the Applied-Virology group for their constructive criticism on the manuscript. The haemophilia gene-therapy programme is supported by The Netherlands Organization for Scientific Research (NWO) and by the Dutch Praeventie Fonds.

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