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Gene transfer into muscle for the treatment of muscular dystrophy and haemophilia

Review Article

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Summary

Muscle has proven to be an appropriate expression system for genes, the product of which is required in the general circulation as well as for muscle genes per se. This review deals with the design of the gene constructs including the vectors and the regulatory elements required for optimisation of expression following introduction of the relevant cDNA by intramuscular injection. The relative merits and problems associated with each type of vector including the immunogenic responses they elicit are discussed. Duchenne muscular dystrophy is used to illustrate the problems associated with gene therapy for a disease in which a muscle protein is defective or missing whilst haemophilia is chosen as an example of how a systemic protein, Factor VIII or IX, may be produced at low constitutive levels in muscle rather than liver.

I. Introduction

The available evidence shows that skeletal muscle is an appropriate target tissue for the transfer of DNA for the treatment of a number of diseases. These include those in which a systemic protein is absent or defective as well as muscle diseases per se such as Duchenne muscular dystrophy. It has also been shown that muscle is also an appropriate target tissue for the introduction of vaccine DNA although the requirements for effective gene therapy is not to elicit an immune response. This review deals, therefore, with the use of muscle as a target tissue for gene therapy for the muscle dystrophies in which the defect is myogenic and for the haemophilias in which there is a requirement to produce steady systemic levels of a particular clotting factor that is defective or missing. Direct intramuscular injection of plasmid DNA was shown by Jon Wolff's group (Wolff et al, 1990) to result in a small percentage of the fibres taking up and expressing the cDNA. This expression has been shown to persist for a considerable time (Wolff et al, 1992). Levels of expression were improved by using different regulatory sequences to drive the expression of the introduced cDNA (Hansen et al, 1991; Novo et al, 1995; Skarli et al, 1998) and by introducing the plasmid constructs into young muscle (Wells and Goldspink 1992). Plasmids are very useful for transfecting muscle by intramuscular injection. However, the possibilities of using other vectors are discussed. The review also deals with the immune response to the vectors as well as the gene product of the introduced cDNA as this is one of the crucial issues in gene therapy.

II. Design of vectors and gene constructs for appropriate expression in muscle

In any gene therapy protocol one needs to consider the construction of the gene cDNA or genomic DNA to be transferred, the vector backbone, as well as host factors in determining immune responses. Consideration needs to be given to these individual components since any one of them can influence the outcome or therapeutic efficacy of a gene therapy approach. In this review these factors will be discussed. However, it is clear that additional factors such as the host immune status (Michou et al, 1997), dose of vector (Svenson et al, 1997), and mode of delivery all

affect the effector immune response but are beyond the scope of this review.

A. Gene constructs for intramuscular injection

For intramuscular injection consideration needs to be given to whether the desired product is secretable or non-secretable. For exocytosis from the muscle fibres it is essential to include an appropriate signal sequence. Also the antigen presentation to the immune system of these responses will differ depending on which MHC pathway is involved. It is known that MHC class I will present intracellularly derived peptides whereas secreted proteins may be processed via MHC II pathways. Most of the early published studies were performed with non-secretable reporter genes such as lacZ whose gene product (ß galactosidase) is non-secretable. Another problem that hampered early gene therapy studies was the availability of species-specific transgenes; most of the available cloned transgenes for early studies were human and these were tested in non-human animal hosts. Recently, many more animal cDNA`s have been cloned which will allow appropriate testing of gene therapy strategies in the homologous animal.

B. Regulatory elements

For effective gene transfer into muscle, tissue-specific expression of the introduced gene is desirable. This can be achieved by the use of promoters and enhancers specific for the tissue of interest. In the case of muscle, creatine kinase (Wang et al, 1997), α-actin (Bergsman et al, 1986; Draghia-Akli et al, 1997), and myosin heavy chain (Skarli et al, 1998) have been used. The necessity for the development of vector bearing muscle-specific regulatory elements is further emphasised by recent studies which have shown that intramuscular injection of genes results in the uptake of a proportion of the vector by the motoneurones (Keir et al, 1995; Ribota et al, 1997). Engineered genes that are under the control of myosin DNA regulatory elements have several potential advantages. Myosin isoforms are differentially regulated by physical activity and by hormones and this, therefore, offers the possibility for developing inducible vectors. Also muscle-specific elements used to drive DNA are more likely perceived as safer than viral promoters.

C. The vector

The choice of vector is crucial for the outcome of the immune response; construction of vectors that addressed these problems in early studies involved non-viral as well as viral delivery methods.

1. Non-viral delivery: plasmid approach

For non-viral vectors, such as injection of naked plasmid DNA, there is now evidence to suggest that the actual plasmid backbone acts as an adjuvant to the stimulation of a host immune response. It is clear that this immune response is both cell mediated and humoural. The immune response is associated with the presence of non-coding immunostimulatory sequences (ISS) within the plasmid backbone which are centred around hypomethylated CpG base pairs (Sato et al, 1996). These motifs are encountered frequently in eukaryotic DNA. Less than 5% of the cytosines in the CpG base pairs in prokaryotic DNA are methylated, but the frequency of methylated cytosines in CpG base pairs in eukaryotic DNA is 70-90% (Tighe et al, 1997). These hypomethylated motifs rapidly stimulate the innate immune response with production of IFN-γ by NK cells and IFN-α and β, IL-12 and IL-18 by macrophages. This response is important phylogenetically as it is the host's first line of defense against bacterial infection. In the adaptive immune response bacterial DNA favours the development of a TH_1-driven response and secretion of IFN-γ favours immunoglobulin class switching to the IgG2a subtype. Because of the strong and persistence cell mediated and humoural immune response to the plasmid backbone and encoded transgene, the use of DNA vaccines has an enormous potential against infectious diseases, allergy, and cancer. However, for use in gene transfer to generate a secretable protein this strong immune response is counter productive (see below). Therefore, plasmid vectors for gene therapy strategies should be designed to lack these ISS sequences. Nevertheless, for vaccine-based strategies the incorporation of these ISS endowed with adjuvant properties would appear beneficial.

2. Viral delivery: adenoviral vectors

The first vectors to be tested in gene therapy approaches were based on the adenovirus; adenoviral vectors still have advantages and are currently being used particularly in the field of haemophilia. Advantages include the ease with which they can be prepared in high titre, their wide host range and their ability to transduce non-dividing target cell such as liver and muscle. However, one of the early problems encountered with the use of these vectors was a rapid decline in gene expression although the early expression levels attained were high; re-administration of the adenoviral vector was precluded because of immunogenicity; expression of the transgene was finally lost. Subsequent experiments revealed that the decline in expression appears to arise from the elimination of the therapeutically-transduced cells (both muscle and liver) by the host immune response (Dai et al, 1995, Yang et al, 1994a, Yang et al, 1994b). This resulted from a CTL (cytotoxic T lymphocyte) response induced by the adenoviral proteins. The immune response also appears to mediate the failure of expression following repeated administration of the vector. Because of these problems a number of immunomodulatory strategies are being developed. Engineering of less immunogenic adenoviral vectors (so called "gutted" adenoviral vectors) is being developed so that the vectors contain only the minimal cis elements required for replication and packaging but are

devoid of any regions encoding viral proteins (Fisher et al, 1996, Hardy et al, 1997).

Immunomodulatory strategies have involved the use of immunosuppressive drugs such as cylophosphamide and cyclosporin, and the blocking of costimulatory pathways involved in T cell activation (Kay et al, 1995). These strategies have met with varying degrees of success but once optimisation can be reached it would be a major advance in adenovirus-mediated gene transfer.

Because of the problems encountered with these two approaches, investigators have sought alternative vectors for use in gene transfer strategies in muscle. One such candidate vector for gene transfer is the adeno-associated virus (AAV). Significantly, AAV-mediated gene transfer in muscle is not highly immunogenic, as is the case for adenovirus.

III. Muscular dystrophies

A. Background and difficulties

Duchenne muscular dystrophy (DMD) is a sex linked hereditary disease which afflicts about 1 in 3200 young boys associated with progressive wasting of the muscles. The pathological defects in DMD, which are believed to result from dystrophin deficiency, are profound and widespread affecting in particular skeletal and cardiac muscle. DMD is lethal in the 2nd or 3rd decade of life but its debilitating effects are seen as early as 3 years of age. When affected boys become about 12 years old, their developing skeletal deformations and progressing muscle weakness confines them to the wheelchair. Subsequent heroic surgical procedures include spine immobilisation so that they can still sit up in a chair and thus improve their quality of life to some extent. The large dystrophin gene is susceptible to *de novo* mutations (>30%) and this makes complete disease prevention impossible. Therefore, effective therapy for DMD, remains a prime goal of research in this area.

The autosomal dystrophies including limb girdle have recently been shown to arise from mutations in the genes encoding the other components of the dystrophin complex. These include the dystroglycans and sarcoglycans and also the extracellular proteins merosin, the form of laminin found in muscle and peripheral nerve (Sunada et al, 1994, Xu et al, 1994). Dystroglycans and sarcoglycans are linked to glycoproteins that in turn are attached to the dystrophin which is associated with the membrane of the muscle fibre. The fact that the dystrophin itself (Milner et al, 1993, Shemanko et al, 1995) is phosphorylated and its associated glycoproteins contain numerous potential phosphorylation sites, strongly suggests that the whole complex is involved in gene activation. This is likely to involve a growth factor gene that in turn uses an established pathway to activate the transcription of a range of structural genes.

Possibilities exist for replacing the proteins that are defective or not expressed in each of the different types of dystrophy. Because of the severity of the disease virtually all the dystrophy gene therapy work has been aimed at Duchenne muscular dystrophy. Apart from the large size of the dystrophin gene and its cDNA, there have been difficulties due to the immune response generated by the introduced gene and/or the vector. When a reasonably mature individual lacks a particular protein and is then exposed to it, immune responses are generated. In patients, these proteins even though they are intracellular, are regarded as foreign. This was highlighted by a case in which a patient with Becker muscular dystrophy received a heart transplant and antibodies directed to parts of the dystrophin protein were detected in the blood shortly afterwards (Britter et al, 1995). Becker is a milder form of muscular dystrophy in which a truncated form of dystrophin is produced. However, there was a marked immune response to the domains in the normal dystrophin of the transplanted heart, which are not present in the endogenous dystrophin of this Becker patient. This emphasised that even particular domains of intracellular proteins will be regarded as foreign if they have never been produced in that individual. As with other gene therapy procedures there have been problems with the immunogenicity of the vectors used.

Gene transfer for Duchenne muscular dystrophy poses additional difficulties. The disease affects every skeletal muscle in the body as well as the heart and the respiratory muscles. Therefore the transferred gene has to be taken up and expressed by a large number of muscles. Ideally, this would be achieved by systemic administration of DNA but this presents a number of problems. The rate of DNA uptake is different in different tissues and is probably different in different muscles depending on factors such as the amount of connective tissue present and vascular density. Other factors to be considered are DNA loss via liver uptake, and DNA uptake by other tissues. Most studies on systemic administration of DNA have used DNA-liposome complexes. Liposomes may increase the uptake but their lipid components may also be toxic especially to the kidney and heart (Wright et al, 1998).

The dystrophin gene is very complex and produces a number of alternatively spliced transcripts that are translated into functional proteins. Transcription of different isoforms is regulated by eight promoters and is a developmentally regulated and tissue-specific process (Fabbrizio et al, 1994). There are three long isoforms specific to skeletal muscle, heart and Purkinje cells of the cerebellum where the cDNA is 14 kb long. Most gene transfer vectors have a DNA insert limit well below this size. This has led to efforts to transduce the muscle fibres with truncated forms of the gene, for example a dystrophin minigene that restores part of the function (see below).

Two types of viral vectors have been mainly used in gene transfer studies for muscular dystrophies. Retroviral vectors have been initially used but their efficiency *in vivo* is extremely limited because their uptake by cells requires mitotic division. Therefore their use in muscular dystrophy gene therapy has been limited to myoblast transfer studies (Fassati et al, 1997). More recently research has focused on the use of adenoviral vectors which are taken up very efficiently by muscle fibres (Ragot et al, 1994). However,

first-generation adenoviral vectors induced a cellular immune and inflammatory response that precluded long term expression of the gene (Stratford-Perricaudet et al, 1990; Li et al, 1993). This immune response was thought to arise from the presence of viral sequences in the vector. Recently a new adenoviral vector has been developed with a capacity for 28kb of foreign DNA. This vector is devoid of virtually all viral coding sequences and has been successfully used to transduce fibres in vivo (Clemens et al, 1996; Kochanek et al, 1996; Chen et al, 1997; Floyd et al, 1998).

A number of other viral vectors have been used in gene transfer studies in skeletal muscle. Of these adeno-associated viral constructs have bee shown to be effective. However, their limited capacity for foreign DNA (about 4.5kb) makes them unsuitable for use with dystrophin. However they may be suitable for other forms of gene transfer as for example for haemophilia B. Herpes simplex virus has also been used in muscle but uptake by muscle fibres is very limited (for review see Huard et al, 1997).

One way to increase uptake by muscle fibres is to damage the muscle by inducing necrosis and regeneration by chemical means. Various agents have been used such as bupivacaine (Davis et al, 1993), notexin, and barium chloride. It has been shown that regenerating muscle exhibits increased uptake of DNA which is taken up mainly by the newly-formed fibres. The use of viral vectors is also limited by safety factors. Since helper virus is used to produce the virus in the encapsulated form that can be taken up by cells, the construct to be transferred has to be highly purified. This is difficult to achieve especially on a large scale and there is always the possibility of contamination by helper virus. In addition recombinant events may occur which could lead to the activation of oncogenes; this is especially important when regenerating muscle models are used.

Another approach for the treatment of muscular dystrophy has been the use of isolated satellite cells stably transformed with dystrophin genes that are then reintroduced into the host organism. This approach is hindered by the fact that there is limited integration between the introduced cells and the existing muscle fibres, by the development of immune responses and the limited penetration of satellite cells though muscle. There have been a number of studies on animal models (Rando and Blau, 1994; Rando et al, 1995) but clinical trials (Miller et al, 1997) have shown that there is a negligible therapeutic value in this approach.

B. Alternatives to introducing the full length dystrophin cDNA.

Several laboratories, including our own, have used mini-dystrophin genes (Wells et al, 1992). Using transgenic biology methods we introduced a human Becker type minigene (England et al, 1990) into the mdx mouse which suffers from a dystrophin deficiency dystrophy. This resulted in the expression of dystrophin (albeit a truncated form) and a marked amelioration of the symptoms. These included a significant reduction in serum CPK levels and in the histopathological changes normally associated with the form of dystrophy. Later, the group of Jeffrey Chamberlain (Cox et al, 1993; Corrado et al, 1994) introduced different lengths of the dystrophin cDNA into mdx mice to define targeting to the membrane and levels of expression. Apparently the full length cDNA is required for good expression and, therefore, it is difficult to see how the immunological response can be circumvented. Further transgenic experiments are being carried out by this group to determine which domains of the dystrophin protein are strategic and which are immunogenic.

Another alternative strategy has been pursued in which there is an attempt to introduce a substitute protein that is related to dystrophin and which is expressed in muscle early in development. Recently a related protein called utrophin has been discovered which appears to have arisen during evolution by duplication of the same gene as dystrophin. From this point of view of gene therapy, the upregulation of utrophin seems to offer an alternative strategy particularly as it is expressed before dystrophin in dystrophic as well as normal muscle. Hence, the same immunological problems do not apply. Transgenic experiments in which utrophin has been over expressed in mdx mice (Tinsley et al, 1996) indicate that it may function as a substitute for dystrophin in protecting muscle fibres from accumulated cell damage and cell death. Utrophin is also associated with dystroglycans and seems to have a similar function as dystrophin. It is reasonable to predict that is also involved in the mechanosignalling that is required to prevent the consequences of microdamage i.e. cell death and in the case of muscle, permanent loss of muscle fibres.

C. Down stream treatment for muscular dystrophy

It is now over 10 years since the dystrophin gene was identified. However, we still do not know what function its complex gene product serves apart from, perhaps, stiffening the plasma membrane. The dystrophin gene itself is of a complex structure, the expression of which depends on the cell type. For example it is spliced differently in neuronal cells than in muscle cells. However, the functions of the different length transcripts and gene products are not understood. In skeletal muscle where has received the most attention, the dystrophin protein is known to form part of an elaborate complex that at the N end terminal is attached to actin filaments. At the C terminal end dystrophin is attached to an elaborate array of sarcoglycans, dystroglycans as well as the extracellular matrix via merosin. Tyrosine kinase and nNOS moieties are also associated with the dystrophin complex. It seems inconceivable therefore that this elaborate structure has evolved merely to stiffen the membrane. As with other cytoskeletal systems (Ingber 1997) we believe it is involved in mechanosignalling and gene regulation.

The study of the underlying mechanisms via which cells respond to mechanical stimuli i.e. the link between the mechanical stimulus and gene expression represent a new and important area of cellular physiology (Goldspink and Booth 1992). Various mechanisms have been proposed for the way in which the genes involved in local tissue growth and repair are activated by mechanical signals. These include the production of autocrine growth factors. Because muscle is a mechanical tissue and a tissue in which there is no cell replacement, it is vitally important that local repair is initiated as soon as any microdamage appears. The hypothesis is that the dystrophies are diseases in which the mechanochemical signalling, and hence the local repair mechanisms, are defective.

It has been known for some time that there are local factors as well as systemic factors that regulate tissue growth. The growth hormone /insulin growth factor-1 (GH/IGF-1) axis is the main regulator of tissue mass during early life. Our group (Yang SY et al, 1996) has cloned the cDNA of a splice variant of IGF-1 that is produced by active muscle that appears to be the factor that controls local tissue repair, maintenance and remodelling. From its sequence it can be seen that it is derived from the IGF-1 gene by alternative splicing but it has different exons to the liver isoforms. Unlike the liver isoforms it is not glycosylated, is therefore smaller, probably has a shorter half life and is thus suited for an autocrine/paracrine rather than a systemic mode of action (Yang SY et al, 1996). It has a 52 base insert in the E domain that alters the reading frame of the 3' end. Therefore, this splice variant of IGF-1 is likely to bind to a different protein, e.g. BP5, which only exists in the interstitial tissue spaces of muscle, neuronal tissue and bone. This would be expected to localise its action as it would be unstable in the unbound form which is important as its production would not disturb unduly the glucose homeostasis mechanism. This new growth factor has been called mechano growth factor (MGF) to distinguish it from the liver IGFs that have a systemic mode of action (Goldspink et al, 1996). We have also shown that, in contrast to normal muscle, the mRNA for MGF is not detectable in dystrophic mdx muscles even when subjected to stretch and stretch combined with electrical stimulation (Goldspink et al, 1996). The systemic levels of IGF-1s are mainly controlled by growth hormone in the blood system. Interestingly, it has recently been shown that during intensive exercise most of the circulating IGF-1 is actually derived from the active muscles. Also most of the IGF-1 circulating IGF-1 is actually utilised by the musculature (Brahm et al, 1997). With age, however, the circulating growth hormone and IGF-1 are known to decrease markedly particularly after the initial growth spurt (Rudman et al, 1981). Although IGF-1s produced via growth hormone stimulation are important during early post-natal muscle development, it appears that IGF-1 produced by muscle during exercise becomes more important for the maintenance of muscle mass. The decline in the production by the liver and the inability to supplement it by locally produced IGF-1 is most probably one of the main factors for the progressive inability to repair and maintain muscle and the progressive nature of the dystrophies.

D. Functions of IGF-1 and the need to supplement levels of the autocrine isoform in muscular dystrophy.

As mentioned, there is strong evidence that IGF-1 is important in determining muscle mass and preventing dystrophy as has been deciphered from transgenic mouse experiments. Transgenic mice produced by introduction of the human IGF-1 cDNA under the control of a chicken actin promoter showed elevated muscle but not systemic levels of IGF-1. They also exhibited muscle fibre hypertrophy but with no significant increase in body weight (Coleman et al, 1995). As far as dystrophy is concerned the knockout experiments are very important. These include those in which the IGF-1 gene (Powell-Braxton et al, 1993, Baker et al, 1993) was truncated or the IGF-1 receptor(s) were knocked out (Ayling et al, 1989) and which resulted in early, severe muscular dystrophy. As a consequence these mice died at, or just after, birth. Also, recombinant IGF-1 has been shown to have a marked beneficial affect on murine muscular dystrophy of the dydy dystrophic mouse (Zdanowitz et al, 1995). If the autocrine isoform of IGF-1 (MGF) was used it would be likely to be much more effective in preventing dystrophic changes of loss of muscle tissue. In ongoing experiments, intramuscular injection of a MGF plasmid construct into normal and dystrophic muscles has resulted in an amelioration of the histopathological changes such as the number of central nuclei. Further experiments need to be carried out on younger mice to see if the muscle fibres can be rescued and the progressive nature of the disease halted.

IV. Haemophilias

The haemophilias are used as an example of a disease where muscle tissue may be used to deliver a non-muscle protein systemically. Clotting factors are normally synthesised in the liver, but work by a number investigators has shown that biologically active factor IX can be produced in other cell types including myoblasts (Yao et al, 1992), fibroblasts (Palmer et al, 1989) and endothelial cells (Yao et al, 1991). Thus the choice of the target cell is not limited as long as the clotting factor protein can gain access to the circulation. Haemophilia is a common disease that occurs worldwide. Haemophilia B that is due to factor IX deficiency affects approximately 1 in 10,000 male births; haemophilia A that is caused by factor VIII deficiency is approximately three times more common than haemophilia B.

Even in developed countries the disease may be regarded as life threatening (as it often results in intracranial haemorrhage) without prophylactic treatment. Also chronic morbidity may arise from repeated joint bleeding resulting in joint contractures and deformity. Highly purified serum-derived factor VIII (i.e. free of HIV, B and C hepatitis and prions) is very expensive and for prophylactic treatment it

is prohibitively expensive (about $80,000 per year). No health service can afford to fund these patients except in emergency situations and 80% of the global population of haemophiliacs do not have access to even emergency treatment. Recombinant factor VIII and IX are now available but these are also extremely expensive and not freely available. Concerns still remain over the safety of plasma-derived products. Haemophilia has been widely regarded as a target for gene therapy because it is a well-characterised single gene disorder and only a small correction in clotting level is necessary to significantly improve the bleeding phenotype in severely affected patients. Another major advancement towards treating haemophilia by gene therapy has been the recent development of large and small animal models that accurately mirror the human disease. These models will prove very useful in working up gene therapy protocols in animals before clinical trials are approved for humans. Because of the knowledge accumulated about the molecular biology of the factor IX gene most of the early work in gene transfer for haemophilia has been performed using the factor IX gene. In the following section a non-viral gene delivery approach (plasmid-mediated) and viral delivery approaches in muscle are discussed.

A. Non viral gene delivery: plasmid-mediated approach

Some of the early *in vivo* attempts using muscle as a target cell in our laboratory consisted of direct injection of a plasmid construct encoding the factor VII gene (this was chosen at the time because of availability and ease of manipulation in cloning and expression). The engineered construct consisted of the FVII cDNA under the control of a myosin heavy chain promoter from which negative regulatory elements were removed. A myosin enhancer was also included and this has later been shown to increase the level of expression several fold. The product of the internal gene was detected systemically and shown to have biological activity (at day 4) although was short lived and disappeared by day 7 (Miller et al, 1995). Subsequent follow up experiments revealed that although factor VII expression was detectable at a tissue level, the muscle sections revealed dense inflammatory infiltrates (consisting of CD4 cells, CD8 cells and macrophages) around the areas of tissue expression (Fields et al, 1998a). An antibody was also detected in the serum of the injected animals to the human factor VII antigen. Similar experiments were performed following injection of a plasmid containing the human Factor IX cDNA into mouse muscle and again this demonstrated little plasma elevation of systemic factor antigen but the presence of an antibody isotype IgG2a to the transgene. This would be in keeping with TH_1 driven cell response occurring in the setting of plasmid mediated gene transfer for a secretable protein (Fields et al, 1998b). These experiments illustrated the importance of selecting a species-specific transgene in a gene therapy setting. These experiments also demonstrated that although the concept of *in vivo* gene therapy for haemophilia using muscle is a viable approach, the efficiency of gene transfer is low. Therefore, although expression was demonstrable, plasmid-mediated gene transfer was short lived due to the induction of the host immune response. New evidence suggested that although plasmid mediated-gene transfer is very inefficient for expression of a secretable protein, its immunostimulatory adjuvant effects are highly desirable in various vaccine strategies against infectious and malignant diseases.

B. Viral delivery approaches

1. Retroviral

Many of the initial viral vectors tested for a gene therapy approach in haemophilia B were retroviral. This involved an *ex vivo* approach in transfecting cell lines and subsequently transplanting them back into rodent animal models. Some early success was achieved transfecting murine myoblasts with a retroviral construct expressing factor IX, with factor IX being expressed for up to six months, although at low levels (Dai et al, 1992, Yao et al, 1992). This was presumed to arise from inefficient expression cassettes (Dai et al, 1992) and a cross species specific transgene evoking a host immune response. Studies were then performed in large animal models but the results were less successful with only short-term expression at levels that were clinically insignificant (Verma et al, 1994). Although some limited success was derived from the use of retroviral vectors there were still many problems to be resolved linked to these vectors. These related to low viral copy numbers, requirement for target cell division, size limitation of the transferred gene, and the concern about potential long term safety of these vectors with respect to their tropism, random integration and oncogenic potential.

Because of these early problems with retroviruses investigators began working with adenoviruses; some of the problems associated with adenoviruses were discussed above.

2. Adeno-associated virus (AAV) vectors: a major recent advancement in gene therapy of haemophilia

Recently, AAV vectors have shown great promise in a gene therapy setting of haemophilia using muscle as a target tissue both in small and large animal models of haemophilia B. Several studies have documented that AAV vectors can direct persistent expression of reporter genes in muscle fibres of immune competent animals (Xiao et al, 1996, Kessler et al, 1996, Fisher et al, 1997). One early report documented expression of therapeutic levels of erythropoietin following intramuscular injection of an AAV vector expressing erythropoietin (Xiao et al, 1996). AAV vectors have certain advantages that make them particularly attractive for muscle-directed gene therapy. These include their relative non-pathogenicity in normal individuals (up to 80% of humans are infected with parvovirus), their ability to infect non-dividing cells and a

broad range of host recipients (primates, canine and murine models). Recombinant AAV vectors contain the engineered expression cassette for the transgene flanked only by the inverted terminal repeats and is therefore devoid of any viral coding sequences. Transduction of muscle with recombinant AAV is very efficient, and expression is stable and long-lived without eliciting the cellular immune responses characteristically seen in muscle after adenovirus-mediated gene transduction (Xiao et al, 1996; Snyder et al, 1997). Various *ex vivo* approaches have targeted muscle in the past for factor IX gene expression; it is known that factor IX produced in myotubes *in vitro* is biologically active (Dhawan et al, 1991). With the use of AAV vectors it is now possible in an *in vivo* gene therapy setting to transduce muscle in a highly efficient way. Stable expression of therapeutic plasma levels of human factor IX (1 year) has been demonstrated after intramuscular injection of AAV-factor IX to Rag-1 immunodeficient mice; levels of 4-7% (200-350 ng/ml FIX Ag) of normal levels of FIX in a human being were attained (Herzog et al, 1997a). When this experiment was repeated in an immunocompetent mouse, no factor IX could be detected in mouse plasma due to the presence of an antibody to the human transgene. When this experiment was scaled up to a canine haemophilia B model a similar result was obtained (Monahan et al, 1998); furthermore data from the canine model indicated that antibody formation against the transgene could be avoided if species specific transgene boundaries are not transgressed (Herzog et al, 1997b).

The results achieved so far with these vectors are very encouraging; optimisation of these gene vectors has been achieved in rodent models resulting in persistent expression of therapeutic plasma levels of clotting factor IX with reduced or absent cellular immune responses against the transduced muscle cells. Scale up of these approaches to larger animal models of haemophilia will form the basis for future human clinical trials.

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