Prospects for Gene Therapy by Gene Transfer in Regenerating Skeletal Muscle

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Abstract

Genetic diseases of skeletal muscle, such as the muscular dystrophies, could be cured by somatic gene therapy. In addition, it has been found that skeletal muscle has the capacity to act as a secretory organ and gene transfer into muscle can be considered for a variety of other genetic and non genetic diseases curable by the systemic delivery of recombinant proteins (e.g. hemophilia and congenital hormone deficiencies). However, clinical trials in humans must await the solution of basic problems concerning the efficiency, stability and regulation of expression of transgenes in skeletal muscle. To address these issues we are performing experimental studies aimed at: 1) developing procedures that allow efficient transfection in vivo in muscle tissues using plasmid vectors; 2) identifying tissue-specific regulatory sequences to be inserted in the vectors in order to obtain regulated and stable expression of transgenes; 3) defining the capacity of skeletal muscle fibers to produce and deliver systemically recombinant serum factors. This is a brief progress report on work recently published or underway in our laboratory.

Key words: gene therapy, gene transfer, regenerating skeletal muscle, DNA injection.

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Efficient gene transfer by direct DNA injection in regenerating skeletal muscle

Foreign genes, such as bacterial chloroamphenicol acetyl transferase (CAT) or β-galactosidase (β-gal), can be expressed in skeletal muscle after direct intramuscular injection [13]. This method has been used to transfer dystrophin and minidystrophin genes into skeletal muscles of the dystrophin-deficient *mdx* mouse, using either naked DNA or recombinant viruses [1, 7]. However, the efficiency of gene transfer using plasmid vectors is very low. We have found that much higher levels of transgene expression are obtained after intramuscular DNA injection in injured muscles undergoing a regenerative process. Using different constructs, containing reporter genes under the control of viral promoters, we found that gene transfer into regenerating muscle leads to about 100-fold higher levels of expression compared to normal muscle [11]. This approach can provide a useful system for the study of transgene expression in muscle. We observed that expression levels progressively decrease with time and that immunological reactions apparently triggered by the bacterial gene product coded by the reporter gene lead to elimination of the transfected fiber segments [11]. In subsequent experiments we have been able to demonstrate increased serum levels of anti-β-gal antibodies in rats transfected with β-gal constructs.

Gene transfer into regenerating muscle for promoter analysis. Validation of in vivo transfection techniques aimed at identifying muscle-specific regulatory elements

A critical point for efficient and sustained expression in vivo is related to the type of promoter directing transcription of the transgene. This point is clearly illustrated by studies of Verma's group on human factor IX gene introduced in mouse myoblasts via infection with recombinant retroviruses [4]. When myoblasts were subsequently implanted in hindlimb muscles of recipient mice sustained secretion for over 6 months of human factor IX was obtained with vectors containing the creatine kinase gene enhancer linked to cytomegamovirus promoter. In contrast, in the absence of the muscle-specific enhancer the secretion of the human protein declined to control levels

after 2-4 days, presumably due to inactivation of the viral promoter. The regenerating muscle should provide a suitable system to select and test the relative efficiency of muscle-specific promoter/enhancer combinations.

The function of tissue-specific promoter/enhancer combinations has so far been investigated only by *in vitro* transfection assays and in transgenic animals (see ref. [10]), i.e. under conditions in which gene regulation is not necessarily identical to gene therapy in adult animals. Intramuscular injection of plasmid DNA into regenerating rat skeletal muscle appears to provide an appropriate experimental system for promoter analysis and for identification of factors responsible for the differential expression of specific genes in the various types of muscle fibers, e.g. MHC-β/slow, -2A, -2X and -2B in the corresponding fiber types (see ref. [5]). In collaboration with M. Buckingham and R. Kelly (Inst. Pasteur, Paris) we have transfected a number of constructs containing regulatory sequences of the mouse myosin light chain 1 slow (MLC1s) gene into regenerating fast and slow rat muscles. Reporter gene product is determined 10 days after the induction of muscle regeneration, when the endogenous MLC1s gene is known to be expressed at high levels in the slow soleus but not in the fast extensor digitorum longus muscle. Our preliminary results show that promoter/reporter gene constructs display a higher level of expression in the regenerating soleus like the endogenous MLC1s gene [9]. The expression of the transgenes was also found to be dependent on intact innervation, like that of the endogenous MLC1s gene. Gene transfer into regenerating muscle can thus be used to characterize muscle-specific and nerve-dependent regulatory elements of muscle genes. These regulatory sequences could be used for gene therapy applications, involving both viral or non viral vectors.

Regenerating muscle fibers as potential targets for gene therapy of muscle dystrophies. Progressive decrease of the regenerative capacity of dystrophic muscle

The finding that gene transfer via intramuscular injection of plasmid DNA is markedly increased in regenerating muscle is relevant to gene therapy of muscular dystrophies. Dystrophic muscles are characterized by the presence of numerous regenerating fibers, however the regenerative capacity tends to decrease with age.

In collaboration with D. Parry (Ottawa) we have investigated spontaneous and induced muscle regeneration in normal and dystrophic (dy/dy) mice of different ages, using an anti-embryonic myosin heavy chain antibody that stains specifically regenerating fibers [8]. Numerous labeled regenerating fibers were seen in muscles from young adult dystrophic mice whereas very few labeled fibers were present in old dystrophic muscle. Cold injury was induced by freezing the exposed surface of tibialis and gastrocnemius muscles. Five days after injury a large number of labeled regenerating fibers were identified at the site of the lesion in both young and old normal mice. In contrast, the response of dystrophic muscles varied strikingly according to the age of the animal. Muscle regeneration was massive in young dystrophic mice whereas few and very small myofibers staining for fetal myosin were seen in old dystrophic animals. These findings demonstrate that the regenerative capacity of dystrophic muscle decreases dramatically with age, independently of the inducing stimulus. This occurs also in Duchenne muscular dystrophy. Clonal analyses of myogenic cells from muscles of Duchenne patients have revealed that the replicative capacity of myoblasts decreases markedly with increasing donor age [3, 12]. It is therefore clear that in order to take advantage of the increased gene transfer efficiency in regenerating muscle any gene therapy protocol should be applied during early stages of the dystrophic process, when regenerating fibers are still numerous.

Gene therapy in regenerating muscle for secretion of recombinant serum factors

The notion that skeletal muscle is a secretory organ opens the possibility to use gene transfer in muscle to deliver systemically a variety of serum factors for the treatment of genetic and acquired diseases, such as hemophilia (factor VIII and IX), congenital hormone deficiencies (e.g. growth hormone), anaemias (erithopoietin), emphysema (alpha-1-protreinase inhibitor), and diabetes (insulin). The transfection experiments performed so far have used cultured myoblats that were transfected *in vitro* and subsequently transplanted *in vivo*. We plan to perform similar experiments using vectors that contain muscle-specific promoters and should thus allow more stable transgene expression. In addition, we want to explore the alternative possibility of *in vivo* transfection into regenerating muscle.

Previous *ex vivo* transfection experiments have shown that muscle cells have the capacity to secrete growth hormone (GH). The human GH gene was transfected in C2C12 myoblasts and these cells were subsequently transplanted into mouse skeletal muscle [2, 6]. Serum levels of GH as high as 0.28 ng/ml, i.e. values similar to those measured in normal human volunteers, were detected at 3 wk after intramuscular injection of $6x10^6$ myoblasts transfected with a plasmid containing the human GH gene driven by a viral promoter [2].

We have started similar studies using primary muscle cultures and in preliminary experiments we have been able to transfect cultured muscle cells with a plasmid vector containing the rat GH gene driven by a viral promoter. Significant levels of GH were detected in the culture medium. We will now perform *in vivo* transfection experiments by injecting rat GH cDNA driven by a viral promoter into regenerating muscles. The effect of GH gene therapy will be determined in GH-deficient hypophysectomized rats.

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