REVIEW

Using skeletal muscle as an artificial endocrine tissue

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Abstract

Gene transfer into muscle tissue is currently being developed as a method for the production, secretion and delivery of therapeutic proteins. This methodology has been used to produce a variety of physiologically active proteins and may ultimately be applied to the treatment of several diseases. In this review, we consider several applications of this methodology and discuss approaches for

modulating therapeutic protein production and secretion from muscle, using growth hormone as an example. In addition, factors limiting the effectiveness of muscle gene transfer are also discussed, as these shall determine the efficacy of muscle gene transfer when applied to humans.

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Introduction

The use of skeletal muscle for producing therapeutic proteins following muscle gene transfer (MGT) is a rapidly developing area of gene therapy. In the past 8 years, MGT has progressed from the expression of bacterial reporter genes (Wolff *et al.* 1990), to the production of hormones (Tripathy *et al.* 1994) and neurotrophic factors (Haase *et al.* 1996). This remarkable property of muscle, for producing many different types of protein, has led to its systematic evaluation and while the application of MGT appears promising, little is yet known about medium to long-term safety. In addition, it is important to develop techniques for regulating therapeutic gene expression in muscle tissue, as the controlled release of protein will be a requirement for many clinical applications. Although several of the proteins described in the following sections are not classical hormones, their production in muscle serves as a useful paradigm for secreted proteins in general. This review evaluates the key factors affecting the efficacy and safety of MGT.

Why use muscle for gene transfer?

Several factors make skeletal muscle an attractive site for therapeutic gene expression. First, it is an abundant tissue, making up about 40% of the average adult's body mass and secondly, it is accessible to most of the delivery methods currently used in gene therapy. There is no significant cell

replacement in skeletal muscle tissue (Fischman 1972) and the introduced genes are not constantly lost following mitosis. This results in durable therapeutic protein production in muscle (Yao *et al.* 1991, Danko & Wolff 1994, Tripathy *et al.* 1994).

In addition to skeletal muscle, smooth (Feldman *et al.* 1996) and cardiac muscle tissue (Rothmann *et al.* 1996) are also being investigated as potential sites for therapeutic gene expression, where local delivery of protein is often desirable.

Gene transfer methods

Therapeutic genes are delivered by injection into muscle tissue using several different types of vector, the choice of which depends on the specific application (Gorecki & MacDermot 1997, Vile *et al.* 1998) (Fig. 1). Recombinant viral vectors derived from human adenoviruses and adeno-associated viruses (AAV)/parvoviruses are frequently used for MGT because of their capacity for transfecting large numbers of muscle fibres. However, for the purpose of safety, viral vectors are modified by removal of immunogenic sequences and the regulatory sequences essential for replication (Yang *et al.* 1996, Fisher *et al.* 1997, Yeh & Perricaudet 1997).

Plasmid DNA-based vectors are used for MGT, as these also transfect significant numbers of muscle fibres following intramuscular injection (Davis *et al.* 1993). The efficiency of plasmid DNA uptake by muscle cells can

Figure 1 Three types of muscle gene delivery vector. Step 1. Viral vectors with deletion of all viral protein coding sequences, e.g. adenovirus encoded dystrophin. ITR – terminal repeat, Ψ – packaging signal, BGAL – *E. coli* lacZ reporter gene controlled by human cytomegalovirus promoter, MCK – muscle creatine kinase promoter controlling dystrophin cDNA expression (Kochanek *et al.* 1996). Step 2. Plasmid gene transfer, e.g. plasmid encoded growth hormone-releasing hormone (pGHRH), α ACT – skeletal alpha actin promoter (Draghiaakli *et al.* 1997). Step 3. Cell based gene transfer into muscle, e.g. myoblasts encoding murine erythropoietin (mEpo) cDNA, DP – doxycycline inducible promoter (Bohl *et al.* 1997).

further be increased by combining these DNA molecules with synthetic polymers such as liposomes (Gregoriadis *et al.* 1997) or polyvinyl derivatives (Mumper *et al.* 1998). In addition, other novel methods such as *in vivo* electroporation have been shown to increase both the number of muscle fibres taking up DNA and the plasmid copy number within these fibres (Aihara & Miyazaki 1998). In common with viral vectors, expression of plasmid encoded cDNA is driven using either viral or other eukaryotic transcription elements (Wang *et al.* 1996, Novo *et al.* 1997) (Fig. 1).

A third method for introducing genes into muscle tissue uses genetically modified myoblasts or fibroblasts. Myoblasts are muscle progenitor cells which differentiate to form the multinucleated myotubes found in muscle tissue and these will stably produce a therapeutic protein once incorporated into new muscle fibres. In addition, cells can be genetically modified to express a variety of different genes and this enables a more precise control of therapeutic gene expression (Rivera *et al.* 1996, Bohl *et al.* 1997) (Fig. 2). However, implanted myoblasts and fibroblasts have to be autologous, or the recipient must be given immunosuppressants to prevent rejection of these cells (Pin & Merrifield 1997).

Evaluation of therapeutic gene expression in vitro

Myoblasts can also differentiate into myotubes in tissue culture and these multinucleated cells express many of the

genes which are active in mature muscle fibres. Thus, effective combinations of transcription elements for driving therapeutic gene expression can be determined first *in vitro* (Novo *et al.* 1997). This methodology is also used to confirm that the expressed protein has an appropriate biological activity prior to any *in vivo* study (Tripathy *et al.* 1994, MacColl *et al.* 1998).

Applications of gene transfer in muscle

Following the evaluation of therapeutic gene expression *in vitro*, MGT can then be applied to an appropriate experimental animal model for the disease being studied. Several of these key *in vivo* studies are discussed below.

Erythropoietin insufficiency

The production of the haemopoietic factor, erythropoietin (Epo), in muscle tissue has been suggested as an alternative to the frequent doses of recombinant Epo required to treat severe anaemias resulting from renal failure in humans (Tripathy *et al.* 1994). In mice, both serum Epo levels and haematocrit can be raised by MGT of Epo coding sequence and the dose of Epo produced in muscle tissue is dependent on the amount of vector used. It is also significant that serum Epo levels remain elevated for several months, indicating that the delivery vector is maintained in the muscle tissue for this time. However,

Figure 2 Regulated GH production in muscle. (a) DNA sequences for expression of human GH (hGH) cDNA in human fibrosarcoma cells. DNA binding domain: hCMV – human cytomegalovirus immediate early promoter, E – epitope tag, N – SV40 viral T antigen nuclear localisation sequence, composite of ZFHD1 DNA binding domain with FKBP. Activation domain: FRP and transcription activation domain of p65. Target gene: ZFHD1/min hCMV – tandem ZFHD1 DNA binding sites/minimal CMV promoter for hGH expression. (b) Formation of FRP-rapamycin-FKBP tripartite complex, activation of hGH transcription. (c) Dose-dependent release of hGH into the serum following administration of rapamycin to nude mice implanted with engineered human fibrosarcoma cells. Values are expressed as means $\pm$ S.E.M. (from Rivera *et al.* 1996, with permission from *Nature Medicine*).

the Epo levels observed in these studies were higher than those normally observed under physiological conditions and a persistently high level of haematocrit would not be desirable in humans. Therefore, the production of Epo must be regulated, as under normal physiological conditions an oxygen deficit increases Epo mRNA levels, with subsequent increases in serum Epo levels stimulating erythrocyte production (McGary *et al.* 1997). Methods for regulating Epo gene expression in muscle are discussed in more detail later in this review.

Glomerulonephritis

An overproduction of transforming growth factor beta (TGF β) in the kidney has been associated with the abnormal glomerular matrix deposition occurring during nephritic disease. TGF β activity can be inhibited by the glycoprotein, decorin (Yamaguchi *et al.* 1990), and kidney fibrosis and proteinuria resulting from glomerulonephritis in rats has been attenuated following MGT of a sequence encoding decorin (Isaka *et al.* 1996). Although these results are promising, TGF β also affects other important physiological activities such as wound healing (Border & Noble 1995, Sporn & Roberts 1992) and a general inhibition of TGF β activity by decorin could interfere with these processes. Therefore this proposed treatment for nephritic disease using decorin currently lacks the necessary specificity.

Diabetic neuropathy

Recent studies in both animals and humans demonstrate that neurotrophic factors such as nerve growth factor (NGF) (Rask *et al.* 1998) and insulin-like growth factor (IGF) (Ishii & Lupien 1995, Apfel & Kessler 1996) can reverse the peripheral neuropathy that is associated with diabetes. MGT of IGF-I coding sequence has been used to reduce the progressive motor neuropathy observed in diabetic mice, as a 4-week treatment using plasmid encoded IGF-I increased nerve and muscle activity to the level of non diabetic controls (Coleman *et al.* 1998).

Motor neurone disease

The effects of IGF-I on diabetic neuropathy following MGT suggest that this method could be applied to the treatment of other diseases where severe neural damage occurs. Motor neurone disease (MND) represents a complex group of neurodegenerative diseases which may respond to this approach. A reduction in neurone degeneration, increased innervation, improved motor responses and a significant increase in survival rate were all observed, following MGT of ciliary neurotrophic factor (CNTF) and neurotrophin-3 (NT-3) coding sequences in a murine model of the amyotrophic lateral sclerosis (ALS) variant of MND (Haase *et al.* 1996).

As clinical trials for ALS using recombinant human CNTF protein have so far been unsuccessful due to the poor bioavailability of this factor (Cedarbaum *et al.* 1995), MGT directed expression of CNTF could be used to increase the clinical efficacy of this protein in ALS patients; by directing CNTF expression to the neurones where it is normally produced.

Peripheral vascular disease

MGT could have a significant clinical impact in treating the peripheral vascular diseases which can cause limb ischaemia (Lepantalo & Tukianen 1996). Several studies using animal models for limb ischaemia have demonstrated a positive response to treatment with plasmid encoded vascular endothelial growth factor (pVEGF), a factor which stimulates angiogenesis in muscle tissue (Battegay 1995). MGT of pVEGF into the ischaemic limb caused an increase in capillary density and blood flow (Takeshita *et al.* 1994), suggesting a potential for its application in humans with an advanced disease, where conventional vascularisation is not possible.

However, VEGF is also produced by some types of tumour cell and it inhibits the maturation of dendritic cells (Gabrilovich *et al.* 1996). Inadequate tumour antigen presentation by dendritic cells has been suggested as a potential mechanism for tumour cell immune evasion (Oyama *et al.* 1998). Therefore, the role of this factor in the pathogenesis of cancer must be considered as a potential side effect if VEGF is to be applied to the treatment of peripheral vascular disease.

Single enzymatic defects – Fabry disease

Therapeutic protein can be released constantly following MGT, if promoter elements with continuous activity are used. Patients with systemic diseases requiring expensive enzyme replacement therapy may benefit from this type of protein delivery. For instance, Anderson–Fabry lysosomal storage disease is one such disorder for which MGT is being developed. The inherited α -galactosidase A (α -GAL) deficiency causing Fabry disease results in a widespread accumulation of its glycosphingolipid substrate in most tissues and body fluids and, although enzyme replacement therapy reduces levels of accumulated serum glycosphingolipids, purified human α -GAL is only available in very limited quantities at present (Desnick & Bishop 1995). Recent studies indicate that muscle can produce significant quantities of this enzyme, as Fabry patient fibroblasts take up α -GAL secreted from muscle cells transfected α -GAL coding sequence via its native endocytic pathway (Novo *et al.* 1997). In addition, a more complete model for the treatment of Fabry disease by MGT can now be investigated, with the recent generation of an α -GAL deficient transgenic mouse (Ohshima *et al.* 1996).

DNA vaccines

The final example of MGT describes the development of DNA vaccines and this is currently one of the most intensively studied areas of MGT. DNA vaccines are designed to produce antigens within muscle tissue and, subsequently, antigen production stimulates both cellular and humoral immune responses to pathogenic infections (Waine & McManus 1995). Thus MGT has been used to generate protective immunity against diverse pathogens such as *Mycoplasma pneumoniae* (Barry *et al.* 1995) and Hepatitis B (Davis *et al.* 1996).

DNA vaccines can also stimulate a stronger cell mediated T helper 1 (Th1) based immune response than some conventional vaccines (Roman *et al.* 1997). Another novel application of this methodology is the development of vaccines directed against pathogens which evade immunological neutralisation by antigenic variation; DNA vaccines have been designed to encode several thousands of antigen subunits (Barry *et al.* 1995) and this type of vaccination strategy could be directed against pathogens such as the malarial parasite, *Plasmodium falciparum*.

Regulation of gene expression in muscle

The gene transfer protocols discussed above are designed for continuous release of protein after MGT and, although this may be potentially useful in treating systemic disorders such as Fabry disease, which require a regular supply of α -GAL (Novo *et al.* 1997), this type of delivery may cause side effects in other applications where production of the deficient protein is normally tightly regulated. In the following section, we consider why regulation of therapeutic gene expression is important and, using gene therapy for growth hormone deficiency (GHD) as an example, then discuss how regulation can be achieved in muscle tissue.

Gene therapy for growth hormone deficiency

In humans, as in rodents, GH is released from the pituitary gland in an episodic pulse which is controlled by GH-releasing hormone (GHRH), somatostatin and possibly other GH secretagogues (Bluepajot *et al.* 1998). Many of the features associated with an endogenous GHD, such as low growth rate and serum IGF-I levels, can be corrected in murine models for GHD following injection of GH-secreting modified myoblasts (Al-Hendy *et al.* 1995) or adenoviral encoded GH cDNA (Hahn *et al.* 1996). Plasmid vectors have also been used to produce biologically active GH in differentiated muscle cells, using combinations of viral and muscle-specific transcription for generating optimal levels of this hormone (MacColl *et al.* 1998). Moreover, serum IGF-I levels and growth rate can be increased following intramuscular injection of plasmids

containing either GHRH, or GH coding sequence controlled by a muscle specific promoter (DraghiaAkli *et al.* 1997, Anwer *et al.* 1998). Although muscle transcription elements can restrict gene expression to specific muscle fibre types (Skarli *et al.* 1998), the continuous GH typified by the above methods can cause organ hyperplasia (Hahn *et al.* 1996, Ng *et al.* 1997). Therefore a gene therapy strategy for GHD should ideally possess an inbuilt regulatory element in an effort to mimic the physiological secretion pattern of GH.

Regulated production of growth hormone in muscle

A controlled release of GH from striated muscle has been achieved following implantation of engineered human fibrosarcoma cells into nude mice (Rivera *et al.* 1996) (Fig. 2). These cells express two hybrid proteins, consisting of the ligand binding domains of FKBP12 and FRP, fused with the zinc finger DNA binding domain of ZFBP1 and the transcriptional activation domain of NF- κ B p65 proteins respectively. The human proteins FKBP12 and FRP form a high affinity complex with the immunosuppressant, rapamycin. Thus, following administration of this drug, the fusion derivatives of FKBP12 and FRP expressed in implanted fibroblasts form a tripartite complex, activating GH expression from a ZFHD1-dependent promoter. A dose-dependent rise in serum GH is subsequently observed in response to rapamycin administration (Rivera *et al.* 1996) (Fig. 2).

A similar system, using implanted myoblasts, has been used to modulate serum Epo levels following administration of antibiotic (Bohl *et al.* 1997) and, as a component of antibiotic regulation is currently being incorporated into the design of viral (Hu *et al.* 1997) and plasmid (Liang *et al.* 1996) vectors, this methodology has the potential to deliver an even wider spectrum of biologically active proteins.

Factors influencing the efficacy and safety of gene transfer

We have discussed the use of MGT for producing several different proteins and methods for regulating therapeutic expression within muscle tissue. However, there are several other important factors which will ultimately determine the success rate of MGT when applied to humans.

Efficiency of gene transfer

Where MGT has progressed to human trials, the comparison with the corresponding animal studies has not been favourable, as illustrated by recent developments in gene therapy for Duchenne muscular dystrophy (DMD). Implantation of myoblasts expressing a dystrophin coding

sequence into skeletal muscles of DMD patients was not clinically beneficial to these subjects (Miller *et al.* 1997), in contrast with a similar application in a murine model for DMD which resulted in long-term expression of a dystrophin cDNA (Floyd *et al.* 1997).

The reasons for the failure of dystrophin gene transfer in humans are not clear at present. However, there are distinct anatomical differences between humans and rodents in terms of muscle composition, activity and blood/lymphatic microcirculation. These are factors which contribute to the efficiency of gene transfer and ultimately to the effectiveness of these methods in humans. In addition, the age of the muscle used seems to play an important role in efficiency of gene transfer (Wells & Goldspink 1992, Danko *et al.* 1997).

Immunogenicity of vectors

An immune or an inflammatory response directed against viral, cell and plasmid based delivery vectors can greatly limit the efficacy of MGT, as with other methods of gene transfer (Gorecki & MacDermot 1998). Early studies using an adenoviral based gene therapy to treat cystic fibrosis patients were unsuccessful, due to an inflammatory response directed against the delivery vector in some individuals (Zabner *et al.* 1993). Recent generations of adenoviral vectors contain no viral protein coding sequences and therefore any observed immune response should not result from *de novo* viral protein synthesis (Kochanek *et al.* 1996, Morsy *et al.* 1998). However, over 40 different adenoviral serotypes have been identified in humans (Scadding & Gibbs 1994) and pre-existing antibodies in any individual may limit the efficacy of even a 'gutted' adenoviral vector, as this still requires pre-packaging in a viral protein coat to facilitate entry into the cell (Kochanek *et al.* 1996). This is especially relevant where repeated administration of a delivery vector is necessary for an effective treatment.

AAV vectors are derived from human parvoviruses and are intrinsically less immunogenic than adenoviruses (Fisher *et al.* 1997). However, AAV vectors could be subject to a similar problem as adenoviruses, as a significant proportion of the population will have been exposed to this pathogen (Scadding & Gibbs 1994) and this could also obstruct re-administration of an AAV vector. Moreover, it may be possible to suppress the specific immune responses which limit the re-administration of viral vectors. For instance, activity of the TH2 subset of T helper cells which block a second administration of recombinant adenovirus virus can be diminished by coadministration with interferon-gamma or interleukin-12 (Yang *et al.* 1995).

Plasmid based vectors have also been shown to be immunogenic, as these contain immunostimulatory DNA sequences (ISS) which stimulate both macrophage and T lymphocyte based immune responses (Sato *et al.* 1996, Stacey *et al.* 1996). These ISS also have an adjuvant

property which enhances the effect of DNA vaccines (Roman *et al.* 1997). However, an immune response directed against these ISS will be undesirable in most of the applications of MGT, as a significant proportion of the population have antibodies to bacterial DNA (DeVlam *et al.* 1993). Therefore, it must also be established if a patient's immune system will tolerate plasmid based gene transfer into muscle.

Immunogenicity of expressed transgenes

Although an immune response is the desired consequence of using a DNA vaccine, what is the physical outcome of foreign protein expression in muscle? A common observation following MGT of a heterogeneous protein coding sequence is a peak of expression at 14 days, followed by a decline and then a loss of expression (Wells *et al.* 1997). Work in this laboratory has shown that an immune response is generated against human factor VII after gene transfer into mouse muscle tissue (Miller *et al.* 1995). The subsequent loss of expression in muscle tissue results from an elimination of muscle fibres through a cytotoxic T cell response directed against the immunologically foreign protein (Wells *et al.* 1997).

The expression of divergent proteins in muscle can also break immune tolerance to the corresponding endogenous protein as, for instance, production of human Epo in mouse muscle activates an IgG immune response to endogenous Epo (Tripathy *et al.* 1996). Therefore the primary sequence of a protein must match that of the recipient, to prevent elimination of transfected muscle fibres expressing the protein. Moreover, where a protein is completely absent, as occurs in DMD (Petrof 1998), patients are immunologically naive with respect to the native protein (i.e. dystrophin). This factor could limit the efficacy of a gene therapy for DMD.

Non muscle expression

The safety of MGT also depends on expression of the introduced gene being restricted to the site where the vector was administered. Recent studies demonstrate that both viral and plasmid vectors can be transported retrogradely to motor neurones innervating the injection site, with subsequent expression of the introduced gene in the neuronal cell body (Haase *et al.* 1996, Johnson *et al.* 1998). Although vector uptake by motor neurones might be beneficial in treating MND, it could also result in further damage occurring to the peripheral and central nervous systems and therefore must be investigated further.

The targeting of gene expression to muscle tissue is an important issue and this can be achieved in several ways. First, the use of muscle specific transcription elements in delivery vectors can restrict expression to muscle cells. For instance, the combination of myosin heavy chain promoter and light chain enhancer element used to produce human

factor VII in muscle tissue (Miller *et al.* 1995) directs gene expression to muscle fibres in both the fast tibialis anterior and slow soleus muscles in mice (Skarli *et al.* 1998). Muscle specific expression can also be accomplished by myoblast transfer, as myoblasts will form new muscle fibres at the site of introduction (Pin & Merrifield 1997). In addition, an even more stringent level for controlling therapeutic gene expression in muscle can be achieved by combining both of the above methods with an element of antibiotic regulation. This method has been utilised in the antibiotic-regulated expression of Epo in muscle, as a human desmin promoter with muscle-specific activity drives expression of an antibiotic binding protein and activates expression of Epo within implanted myoblasts (Bohl *et al.* 1997).

Discussion

The examples of MGT discussed in this review demonstrate the potential clinical applications of this methodology and the release of Epo, decorin and IGF-I from muscle show that this tissue can be used as a central site for the production of proteins which have either a local or endocrine effect. However, as with any new drug therapy, the success of MGT depends both on the efficacy and the safety of the methodology used to deliver therapeutic genes into muscle tissue.

The clinical trials for DMD reveal the difficulties inherent in applying MGT to humans. In addition, immune responses to delivery vectors, combined with their tendency to be internalised by neural tissue, are both factors which currently limit the efficiency and safety of MGT. However, the progress currently being made in understanding the mechanisms which underlie vector immunology and transport kinetics may eventually lead to safer methods of gene transfer into muscle. Also, the combination of tissue specific transcription elements with antibiotic regulation can now be used to regulate therapeutic gene expression in skeletal muscle.

Therefore, considering the complex safety and efficacy aspects which must still be resolved, MGT is likely to be applied to the most clinically difficult situations first, i.e. those where no effective treatment exists. Motor neurone and Fabry disease are both candidates for MGT and this methodology may also be applied to the treatment of limb ischaemia resulting from peripheral vascular disease, a cause of major morbidity. These and other applications of MGT have now progressed to the clinical trial stage and the results of these studies will give a clearer indication of where MGT will be most effective.

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