Chapter 16
Gene Electrotransfer to Muscle Tissue: Moving into Clinical Use

Pernille Hojman

Abstract Electrotransfer has been proven as a unique method for gene delivery into tissues. Muscle tissue has been an attractive target due to high efficiency, long-term transgenic expression, and simplicity of the procedure. Gene transfer to muscle is interesting both for vaccination purposes, production of systemic proteins, as well as local correction of myopathies. During the last decade, a large volume of knowledge from rodent studies has accumulated. Presently, the field is moving towards experiments in larger animals and humans where seven clinical trials have been initiated so far. The present review will focus on the knowledge obtained from the preclinical and clinical studies, including the mechanisms and practical considerations when performing muscle electrotransfer both in animals and humans. In addition, the therapeutic applications of muscle electrotransfer will be reviewed.

Keywords Electroporation • Skeletal muscle • Gene therapy • Systemic secretion

Introduction

Gene therapy is a promising tool for the treatment of numerous diseases, and currently more than 1,500 clinical trials on gene therapy have been initiated worldwide [1]. The main obstacle for gene therapy has been the delivery of genes to the target tissue. However, gene transfer by electroporation (DNA electrotransfer) has overcome this barrier and offers high transfection efficiency and improved safety. The clinical perspective of electrotransfer was proven with trials on electrochemotherapy [2], and currently several clinical trials with DNA electrotransfer to muscle tissue are being conducted.

DNA electrotransfer was first reported by Neumann and colleagues in 1982, where they showed that electric pulses could be used for the introduction of plasmid DNA into cells [3]. In 1998, four independent groups consistently demonstrated high transfection levels in vivo using trains of long pulses: Aihara and Miyazaki, and Mir and colleagues in skeletal muscles [4, 5], Rols and colleagues in tumors [6], and Suzuki and colleagues in liver [7]. Since these original studies, a large body of evidence has shown that DNA electrotransfer is efficient in essentially all tissues and in all species (reviewed in [8]).

P. Hojman (✉)
Center of Inflammation and Metabolism at Department of Infectious Diseases, Rigshospitalet, University of Copenhagen, Blegdamsvej 9, 2100 Copenhagen, Denmark
e-mail: pehm@heh.regionh.dk

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DNA electrotransfer is a multistep process, involving (1) electrophoretic migration of DNA towards the plasma membrane, (2) DNA accumulation and interaction with the plasma membrane, (3) intracellular trafficking of the DNA to the nucleus, and (4) gene expression [9]. Steps 1 and 2 are effectively dependent on the electric pulses and the efficiency of these two steps can be controlled through the regulation of the electric parameters. The question of intracellular transport has received much less attention, yet Dean and colleagues have shown that plasmid DNA utilizes the microtubular network for transport from the plasma membrane to the nucleus [10]. Furthermore, the efficacy of gene expression depends on the target cell and the promoter strength of the transgene. Muscle fibers are generally postmitotic long-lived cells, and numerous studies have shown long-term persistent expression in muscles [11, 12].

Skeletal muscles are attractive targets for DNA electrotransfer due to their intrinsic physiological properties such as:

1. Skeletal muscles constitute about 30–40% of normal adult body mass, meaning that the muscle tissue is a large and easily accessible target organ.
2. Skeletal muscle fibers are terminally differentiated; thus individual fibers are designated to persist for a long time, allowing long-term stable transgene expression.
3. Skeletal muscles function as a protein factory, producing large amounts of protein both for maintenance of the contractile apparatus and secretion of myokines (Fig. 16.1).
4. Skeletal muscles have abundant blood supply with an extensive capillary network enfolding the fibers and providing an efficient transport system for carriage of secreted proteins into the circulation.

Nevertheless, muscle tissue is a favorable tissue for DNA electrotransfer as reflected by the large number of publications on muscle electrotransfer. With 27% of all in vivo DNA electrotransfer papers focusing on muscle electrotransfer, muscle tissue is the second most exploited tissue after tumors [9]. That is because DNA electrotransfer to muscle tissue is highly efficient with 200-fold increase in gene expression compared to naked DNA injection [11, 13]. Persistent gene expression with transgene production has been detected for more than a year [11, 12]. In addition, DNA electrotransfer ensures low variability in the level of expression and precise delineation of the transfected tissue by placement of the electrodes [14]. One point, which is noteworthy, is that muscle fibers may produce a different carboxylation and phosphorylation pattern than endogenous production

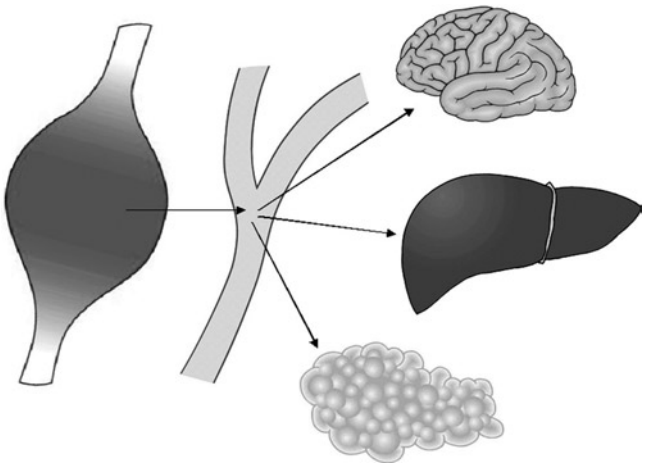

Fig. 16.1 Muscle as an endocrine organ. The muscle has an innate ability to secrete proteins, and the extensive capillary network enfolding the muscle fibers provides an efficient transport system for carrying the produced proteins from the muscle to the whole body including the brain, liver, and adipose tissue

sites, resulting in transgenic produced proteins that are slightly different from endogenous produced proteins [15]. Yet, no functional disturbances have so far been reported for transgenic proteins produced after DNA electrotransfer.

Mechanism of Action

The principles of electroporation are reviewed in the previous chapters, and will not be discussed in details here. However, it should be noted that membrane permeabilization results from position-dependent changes in the transmembrane potential, caused by the external electric field. The changes are described by Schwann's equation ($\Delta V = f\,g(\lambda) r\,E\,cos\,\theta$), which shows that the induced transmembrane potential is proportional to the external electric field (E) and the cell radius (r). The last parameter is quite important for muscle transfection, as muscle fibers are long multinucleated cylinders, indicating that relatively low electric fields are needed for muscle fiber permeabilization. In continuation, satellite cells will not be permeabilized by the same electric parameters as muscle fibers as these cells are considerably smaller in size.

Permeabilization in Muscles

Studies using the inert molecule ^{51}Cr-EDTA have been performed in mice to determine the permeabilization threshold in skeletal muscles. Using eight pulses of 100 μs at 1 Hz resulted in a permeabilization threshold of 530 V/cm, while longer pulses (eight pulses of 20 ms) resulted in a threshold of 100 V/cm. With longer pulses (250 V/cm), the irreversible permeabilization threshold was reached and cells were no longer able to reseal [14, 16]. The studies also showed that in vivo resealing was complete within 9 min. These studies were performed with plate electrodes placed transcutaneuosly where the results demonstrated that as the first cell became irreversible permeabilized, the transfection efficiency dropped dramatically [17].

Rodent muscles have been shown to have higher impedance than muscles in larger species e.g., rabbit, monkey, and pig [18, 19]. This implies that the optimized electric parameters for rodent muscles cannot be directly translated into clinical use; however they may serve as guidelines for selecting pulse parameters.

Pulses for DNA Electrotransfer of Muscles

Large efforts have been put into optimizing and describing the role of the electric pulses. For in vivo studies, square wave pulse generators are generally used as these offer independent control of both amplitude and length of the delivered electric pulses. Most rodent studies have concentrated on electrotransfer with long pulses of ms duration as one of the first muscle papers proved these to be superior in direct comparison with short (100 μs) high voltage pulses [20]. The electric pulses have been shown to act both on the permeabilization of the plasma membrane and on the electrophoretic displacement of the DNA [21, 22]. Permeabilization can be obtained with both short and long electric pulses, given that they are above permeabilization threshold, while the electrophoretic displacement of the DNA requires ms pulses, which can be both above and below permeabilization threshold. This has led to two pulse regiments: trains of ms pulses at 150–200 V/cm or combinations of one short (100 μs) high voltage pulse, followed by long (>100 ms) pulse(s) of low voltage pulses [23].

Monitoring of Tissue Permeabilization in Real Time

The most noticeable, measurable change in tissues undergoing electric pulsing is an increase in tissue conductivity and decrease in tissue resistance due to membrane permeabilization. This changes the electric field distribution and the corresponding current as predicted by Ohm's law *(R (resistance) = V (voltage)/I (current))*. Thus if the resistance drops, while the voltage is maintained constant, the current (flow of electric charge) through the tissue will increase (Fig. 16.2). In larger animals, where the muscle impedance is lower, this current increase is augmented. The current is important for the generation of heat (watt) as predicted by the following equation: W *(watt)* = I^2*R*t (time)*. If the increase in current is not controlled, it might lead to tissue heating with subsequently tissue damage and pain [24].

Regulation of Expression

Intrinsic in the DNA electrotransfer method lies the possibility to control gene expression as it offers several ways of reaching target concentrations in vivo. These include (1) plasmid amount, (2) area of transfection, (3) promoter systems, and/or (4) retransfection [25].

Many studies have shown a clear dose-dependency between the amount of injected plasmid and the level of gene expression. However, as the efficiency of the transcription depends on promoter strength, target cell, and species, no general recommendations of plasmid concentration can be proposed. Nevertheless, plasmid concentrations ranging from 0.05 to 2 μg/μL have typically been used for muscle tissue. It should be noted that excessive plasmid DNA can lead to decreased gene expression probably due to toxic effects of the DNA [25].

A particular feature of DNA electrotransfer is the selective target of the area encompassed by the electrodes, thus electrode placement can be used as a restrictor of the anatomical extent of the transfected muscle tissue. With the current electrode configurations, this area maximally covers 1 cm^3. As much more muscle tissue is available, transfection at multiple sites can increase the total level of transgenic expression. It should be noted that the transgenic product probably is distributed throughout the transfected cells, which for the muscle fibers can be many centimeters.

Inducible promoter systems are available where the level of gene expression can be controlled by adjusting the administered dose of a given inducer drug e.g., doxycycline [25, 26]. These systems have proven to give good control in rodents and monkeys, yet the adverse effects of the inducer drug discourage their clinical use.

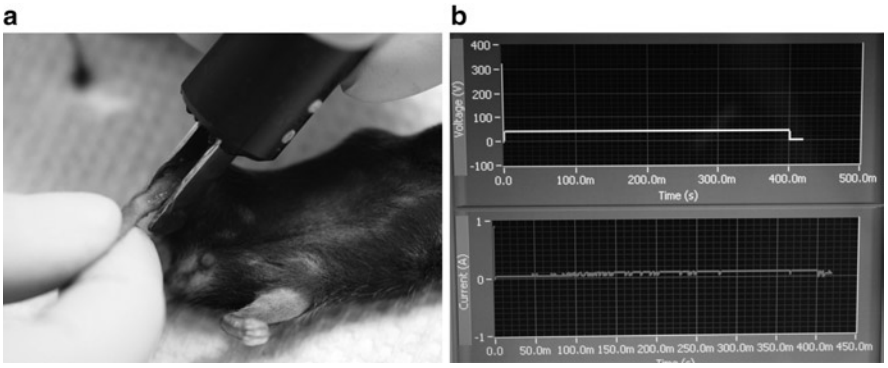

Fig. 16.2 Pulse application to mouse muscle and a representative current trace. (**a**) Application of electric pulses to the tibialis cranialis muscle of a C57Black/C mouse using plate electrodes. (**b**) A representative current trace measured during the application of HV + LV pulses as depicted in (**a**)). The upper curve represents the applied voltage, while the lower curve shows the current measured during pulse application

Finally, DNA electrotransfer shows no immunogenicity towards the vector, meaning that the delivery can be repeated without risk of inducing an immunological reaction. Several studies have shown that retransfer to muscles results in secondary increases in transgenic expression [26–28].

Practical Considerations for DNA Electrotransfer to Muscle

DNA electrotransfer to muscle tissue is basically injection of an isotonic plasmid solution into the muscle, followed by the application of electric pulses by means of electrodes, which are placed around the injection site. Although simple, several points must be considered for successful transfection.

Plasmid Composition

Generally, the plasmid must be composed of a promoter, signal sequences, gene-of-interest, and trafficking signals. Most rodent studies have been conducted using the constitutive active CMV or CAGGS promoters. Experience with larger animal studies suggests that these viral/bacterial promoters are not as efficient in larger animals as seen in rodents. Instead, natural or synthetic muscle-specific promoters including the ubiquitin promoters have been used [29]. Inducible promoter systems are also available, however these rely on administration of an inducer drug e.g., doxycycline to turn on gene expression [30]. Signal sequences helps direct the product to the correct cellular compartment e.g., the secretory pathway [31]. Currently, all studies in larger animals including humans utilize codon optimization to maximize gene expression in the given species. Finally, intracellular trafficking signals including the SV40 enhancer site can facilitate translocation to the nucleus and increase gene expression [32].

Intramuscular Injection

For small animals, the injection volume should be adjusted to the holding capacity of the muscle. Preferentially, the plasmid solution should be injected along the muscle fibers to ensure good distribution along the fibers. For smaller muscles, multiple injections are not recommended as the plasmid might seep out of the previous injection sites.

Electrodes

Application of the electric field results from the voltage applied between two electrodes, thus the electrode design is important for the electric field distribution and in turn, the transfection efficacy. Various electrode designs are available, but electrodes are generally divided into needle or plate electrodes (see also Chap. 5). Plate electrodes can be used to treat superficial tissues and rodent muscles, while needle electrodes can target deeper lying tissues e.g., muscles in larger animals.

For DNA electrotransfer to muscles in small mammals, the most practical way of delivering the electric pulses is by placing plate electrodes around the leg. This prevents surgical interventions and ensures delivery of a homogenous field. Yet good contact between leg and electrodes must be ensured by depilation and application of electrode gel.

For clinical use, needle electrodes of various designs are generally used. Models include a six-needle circular electrode, a hexagonal electrode, and an electrode with two opposing arrays of needles [33]. At least two patented solutions for integrated plasmid injection and needle electrodes exist: Trigrid (Ichor) and Elgen (Inovio) (See Chap. 5). These systems are used in ongoing clinical trials for muscle electrotransfer.

Application of Electric Pulses to Muscles In Vivo

Application of electric pulses to muscles must consider muscle fiber geometry. Placement of the electrodes parallel to the muscle fibers ensures a homogenous field and even transfection in the central region between the two electrodes. In contrast, placement of the electrodes perpendicular to the muscle fibers results in a skewed field distribution with high field strengths near the electrodes and low field strengths in the body of the muscle. In fact, one of the first studies on muscle transfection delivered the electric pulses perpendicular to the muscle fibers, resulting in cell death near the electrodes, transfection in an area, and no changes in the middle of the muscles [13].

Toxicity

The first clinical muscle vaccination studies showed that electrotransfer is well tolerated with no severe side effects and all patients completed the treatment [34]. Applying electric pulses in muscles always results in muscle contraction, even in anesthetized subjects. Also, the frequency of the pulses determines how many muscle contractions are experienced. In the first clinical trials with electrotransfer to the deltoid muscle, the electric pulses were applied without analgesia. In these studies, the patients reported pain for the procedure to be one for needle insertion and four for the electric pulse application on the visual analog pain scale (scale 0–10, with 10 being worst pain imaginable) [35]**.** The pain subsided within minutes. At 48 h after treatment, 97% of the patients reported no more pain. No systemic increases in markers of muscle damage, i.e., creatine kinase (CK) or lactate dehydrogenase (LDH), were observed, and lymphocyte and neutrophil counts remained unchanged throughout the study. All subjects were willing to undergo the procedure again.

Rodent studies have investigated the physiological effect of electrotransfer on muscles. These studies have shown a transient exchange of ions across the sarcolemma, loss of ATP, and a drop in the contractile force immediately after pulse application [36]. These changes are largely dependent on the pulse parameters, as muscle damage and regeneration were only observed for the more rigid high voltage pulse regiments. Likewise, differential gene expression profiles have been investigated after electrotransfer, showing modest changes in gene expression, mainly in genes involved in cytoskeleton and intracellular transport [37]. Regardless of pulse parameters, all studies have shown full muscle recovery after 1–2 weeks, where increased transgenic expression persists.

Therapeutic Use of DNA Electrotransfer to Muscle Tissue

Therapeutic use of DNA electrotransfer to muscle tissue involves vaccination purposes, systemic delivery of therapeutic proteins and local intramuscular expression. The clinical advances have so far been made within DNA vaccines to muscle, where a large preclinical literature exists exploring systemic release of therapeutic proteins after DNA electrotransfer to muscle tissue. The ongoing clinical trials are summarized in Table 16.1, while examples of different therapeutic applications of muscle electrotransfer are discussed below.

Table 16.1 Ongoing clinical trials using muscle electrotransfer

Condition	Status as of August 2009	Enrolled	Primary outcome	Intervention	Clinical Trial.gov identifier	Sponsor
Prostate cancer	Open		A phase I/II trial of DNA vaccine with a PSMA27/pDom fusion gene given through intramuscular injection in HLA A2+ patients with prostate carcinomas with or without electroporation	Biological: prostate specific membrane antigen (PSMA)/pdom fusion gene Device: Medpulser DDS device	UK-112	Inovio biomedical corporation
Papillomavirus infections	Open	24	Safety and tolerability of escalating doses of VGX-3100, administered by IM injection with EP to adult female subjects postsurgical or ablative treatment of grade 2 or 3 CIN as adjuvant treatment	Biological: VGX-3100, E6 and E7 proteins of HPV subtypes 16 and 18 Device: CELLECTRA constant current device	NCT00685412	VGX pharmaceuticals, Inc.
Chronic hepatitis C virus infections	Open	12	Safety and tolerability of electroporation mediated IM delivery of CHRONVAC-C® in chronically HCV infected, treatment naive patients with low viral load	Biological: CHRONVAC-C® Device: Medpulser DDS device	NCT00563173	Tripep AB
Malignant melanoma	Open	25	Safety and feasibility of electroporation mediated intramuscular delivery of a mouse tyrosinase plasmid DNA vaccine in patients with stage IIB, IIC, III, or IV melanoma	Biological: xenogeneic tyrosinase DNA vaccine Device: TriGrid delivery system	NCT00471133	Ichor medical systems incorporated
HIV Infections	Open, accrual completed	40	Safety of an intramuscular prime and boost injection of the ADVAX DNA-based HIV vaccine via TriGrid™ electroporation at three dosing levels	Biological: ADVAX HIV DNA vaccine Device: TriGrid™ delivery system	NCT00545987	Rockefeller university
Healthy adults	Completed	24	Assessment of the tolerability of the MedPulser DDS device	Biological: V930 Device: Medpulser DDS device	NCT00721461	Merck

DNA Vaccination

DNA vaccination by electrotransfer is extensively reviewed in Chap. 18, and the results of the different studies will therefore not be covered in this chapter. However, it is noteworthy that the role of the electric pulses has additional roles in DNA vaccination compared to transfection studies. The role of the electric pulses are both to increase the number of transfected cells and induce mild tissue damage with local inflammation, and increased recruitment of antigen-presenting cells and the activation of danger signals. Thus, electric parameters are typically more rigid than those used for regular DNA electrotransfer.

Systemic Delivery of Therapeutic Proteins

A potentially large benefit of muscle electrotransfer is systemic secretion of therapeutic proteins. Muscles produce and secrete endocrine factors such as interleukin-6, interleukin-8, and leukemia inhibitory factor (LIF) during exercise, proving their potential as exogenous protein factories [38]. Typical examples of potential therapeutic proteins, which have been electrotransferred to muscle tissue, include anticancer agents, hematopoietic growth factors, and metabolic proteins.

One molecule, which is close to clinical testing for systemic release, is GHRH. GHRH stimulates the growth hormone axis. Overexpression of GHRH in muscles has been shown to ensure maintenance of the pulsatile release of GH and respond to endogenous feedback regulation. A number of studies have investigated the role of GHRH in anemia and cachexia associated with cancer and renal failure. In these studies with cancer-bearing or old dogs, long-term expression of transgenic GHRH is associated with increased weight, restoration of activity levels and exercise tolerance, and improvement in hematological factors, proving a role for GHRH in treatment of the catabolic process associated with aging and cancer.

The single most studied molecule for muscle electrotransfer is erythropoietin (EPO) probably due to its easy detection and high efficiency [28, 39–42]. A small amount of EPO is enough to generate physiological effects. In fact, transferring as little as 0.5 μg of plasmid into one muscle results in large increases in the hemoglobin levels in mice and 20-fold increases in circulating EPO [25, 26]. The clinical potential of EPO electrotransfer has been proven in beta-thalessemic mice, where EPO electrotransfer results in both increases in hemoglobin levels and correction of erythrocytes phenotype with increased erythrocyte lifespan [27].

Other potential hematopoietic candidates include factor VIII [43] and IX [44], which have been tested for correction of hemophilia A and B, respectively in mice and dogs. In these studies, as little as 0.5–2% of normal protein levels were enough to reverse the clinical phenotype. This has made these diseases attractive for gene therapy and trials with other delivery methods that have been going on for years, while electrotransfer could be a possible alternative.

A large focus has been on generating muscle-secreted anticancer agents such as interleukins (IL-12) [20, 45, 46], interferons (IFN-α) [47–49], and antiangiogenic factors (metargidin) [50, 51]. All these agents have shown efficient antitumor effect in rodent studies with regression of transplanted tumor and suppression of metastases with foreseen clinical advances.

In addition to the above-mentioned candidates, a large number of molecules have been electrotransferred to study their potential in treatment of disorders like diabetes, ischemia, atherosclerosis, neuropathy, and autoimmune diseases. These have been reviewed in detail in [52] and [53].

DNA Electrotransfer of Genes with Local Effect

Among muscle-specific diseases, the myopathies, e.g., Duchenne muscular dystrophy, are the most pronounced. The most severe dystrophies are characterized by defects in the dystrophin gene, which encodes a cellular scaffolding protein. Several preclinical studies have electrotransferred full-length dystrophin (>17 kb) or shorter versions of the gene, minidystrophin or microdystrophin, into the dystrophic muscles, and found dystrophin formation at the plasma membrane increased muscle fiber strength and improved motor function [54–56]. A different approach to treatment is electrotransfer of oligonucleotides, which can introduce exon shipping and alternative splicing, resulting in a functional dystrophin protein [57]. These studies have also found dystrophin formation at the plasma membrane, and increase muscle fiber robustness and strength.

In the last couple of years, many groups have taken up DNA electrotransfer as a research tool for studying the biological effects of proteins in muscles. Examples include electrotransfer of IGF-1 [58–63] and myogenin [64] for studying muscle regeneration after injury. Other examples of muscle processes, which have been studied by overexpression of mediator molecules, include glucose uptake [65, 66], lipid metabolism [67–69], muscle fiber composition [70, 71], and muscle hypertrophy [72].

Conclusion

Muscle electrotransfer is the nonviral transfer of genes into muscle with high efficiency. It has the advantages of being fast, easy to perform, cost-efficient, and nonimmunological. In this chapter, the mechanisms for muscle electrotransfer and practical considerations when transfecting both animals and humans have been covered in details, showing the large amount of knowledge on muscle electrotransfer, which has been generated during the last decade. The therapeutic perspectives of muscle electrotransfer ranges from the already initiated clinical trials on DNA vaccination to trials utilizing the muscle's potential to act as an endocrine organ, secreting proteins at therapeutic concentrations for treatment of e.g., anemia, inflammatory diseases, and cancer.

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