High Level of Gene Transfer into Adult Skeletal Muscle by In Vivo Direct Electroporation

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Abstract

Gene delivery to adult skeletal muscle is a promising strategy for myofiber signal transduction studies and for the treatment of muscle disorders. Among the nonviral techniques for gene transfer in vivo, electric field applied to the direct injection of plasmid DNA into muscle is safe, inexpensive and simple. However, present DNA delivery technologies should be improved with regard to the level of expression and efficiency of transfection especially when compare to viral-mediated gene transfer. We described a method for a high efficiency transfection in adult skeletal muscle fibers using low amount of cDNA (15 μg) and minimal muscle injury which permits in vitro assay for cell biology. A snap25GFP gene-encoding plasmid was used to assess the conditions of best gene transfer with minimal muscle injury. We use low electric field to target plasmid DNA into skeletal muscles of mice. Quantification of GFP fusion proteins showed that 80 ± 8% of adult skeletal myofibers is transfected after DNA injection. Muscle injury is minimal since regenerating fibers are absent and inflammatory response is transient. Confocal microscopy of transfected fibers showed that the products are localized in the expected subcellular organelle and that the product is expressed along the entire fiber. Moreover transfected fiber are isolated and placed in cell culture for several days. In vitro real-time confocal microscopy and cell biology assays could be applied to transfected adult fibers. DNA electrotransfer in muscle may have broad application in gene therapy and in developmental, physiological and pharmacological studies.

Key words: electroporation, gene transfer, skeletal muscle.

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Nonviral gene transfer for gene therapy is a rapidly expanding field [3, 4, 9]. Tissues for which highly efficient gene transfer is sought include tumors, various epithelia or endothelia, and organs such as the liver, heart, or brain. Special interest has been devoted to gene delivery to skeletal muscle fibers, e.g., for the correction of myopathies such as Duchenne's muscular dystrophy [8], for the local secretion of angiogenic or neurotrophic factors, and also for vaccination [7].

The postmitotic nature and longevity of myofibers permits the stable expression of transfected genes. For this reason special interest has been devoted to gene delivery to skeletal muscle fibers, e.g., for the correction of myopathies as dystrophies, for the systemic secretion of therapeutic proteins as an endocrine organ, for the creation of an immune privilege tissue. Another exciting application is the characterization of signal transduction in adult skeletal muscle fiber during physiological and pathological conditions such as atrophy, in order to develop new pharmacological approaches. The major draw back is that adult skeletal myofiber is extremely resistant to the standard protocol of DNA delivery in *in vitro* experiments. Despite the impossibility to target genes into myofiber *in vitro*, the direct injection of plasmid DNA, *in vivo*, into either adult or regenerating muscle is safe, inexpensive and simple. However, there are several major limitations as interindividual variability of gene expression and the low efficiency.

Among the nonviral techniques for gene transfer in vivo, electric field applied to the direct injection of plasmid DNA into muscle is a novel, safe, and simple approach. However, present DNA delivery technologies should be improved with regard to the level of expression and efficiency of transfection especially when compared to viral-mediated gene transfer. Actually electroporation on skeletal muscle for gene transfer has recently been applied using a broad spectrum of different conditions, e.g., regenerating muscle [1] or adult muscle [6], high voltage [6, 10] or low voltage field [1, 2, 5], needles electrodes [1, 5] or external plaques [6].

However no studies describe the percentage of transfected fibers, if the fibers are adult or regenerating, if the gene is expressed along the entire fiber and if this method is reliable for *in vitro* studies on subcellular localization. We described a method for a high efficiency transfection in adult skeletal muscle fibers using low amount of cDNA (15µg) and minimal muscle injury which permits in vitro assay for cell biology. A snap25GFP gene-encoding plasmid was used to assess the conditions of best gene transfer with minimal muscle injury.

DNA electrotransfer in muscle may have broad application in gene therapy and in physiological, pharmacological, and developmental studies.

Materials and Methods

Plasmids and DNA preparation

The plasmids VR1012 (pCMV-Snap25GFP, a gift of Prof. T. Pozzan), pEGFP C-1 (pCMV-Cytochrome c-EGFP, a gift of Dr. D. Green), containing the cytomegalovirus (CMV) promoter, were grown in DH5α and purified using the Plasmid Maxi Kit (Qiagen, Crawley UK) following the supplier protocol. Identity was confirmed by agarose gel electrophoresis of both uncut and restriction digested plasmids. Contamination with RNA was absent and all the plasmids were present as covalently closed circles.

Tibialis anterior muscle transfection by electric field

In vivo experiments were carried out on 10-week-old CD1 mice. Mouse tibialis anterior (TA) muscles were exposed by a short incision and DNA (0.06-20 µg) in 50 µl of 0.9% NaCl was injected with a Hamilton syringe in a proximal to distal direction. Then, two spatula like electrodes (0,5 cm-large 2 cm long) were placed at each side of the muscle and electric pulses were delivered. Five electric pulses with a fixed pulse duration of 20 ms and an interval of 200 ms were delivered using an electric pulse generator (Electro Square porator ECM 830, BTX, San Diego, CA). The ratio of applied voltage to electrode distance was 50 V/cm. For comparison, preliminary experiments were performed on mouse TA muscles electroporated either by external stainless steel plate electrodes or by needle electrodes. Mice were sacrificed at different times (2, 3, 4, 5, 7, 8, 10, 20, 30 days) and muscles (N>8 for each point) were removed, frozen in liquid nitrogen and stored at -80°C.

Flexor digitorum brevis muscle transfection by electric field

For studying the subcellular distribution of transfected genes we injected flexor digitorum brevis (FDB) muscle (a well-characterized fast muscle) with cytochrome c-GFP and snap25GFP constructs (15 µg DNA in 0.9% NaCl). Three electric pulses with a fixed pulse duration of 20 ms and an interval of 200 ms were delivered using an electric pulse generator (Electro Square porator ECM 830, BTX, San Diego, CA). The ratio of applied voltage to electrode distance was 50 V/cm.

Isolation of adult skeletal myofiber from muscle (FDB) and FDB cultures

After seven days mice were sacrificed and FDB muscles were removed and adult fibers were dissociated from FDB muscles. After removing the outer connective, the muscle is immersed in a relaxing buffer (0.1 M KCl, 5 mM EDTA, 5 mM $MgCl_2$, 3 mM BDM, 0.25 mM DTT, 10 mM histidine pH 7.8) and then in Tyrode solution (Sigma) containing 0.3% collagenase (type I, Sigma) and 10 % FCS and incubated for 60 min at 0-4°C and then 60 min at 37°C, mixing every 15 min. Muscle bolus was then forced trough a wide-mouth Pasteur pipette to release the single fibers. Dissociated fibers were then transferred onto a sterile glass coverslip in a petri dish and bathed with a Tyrode solution for 1 h at 37°C to allow the adhesion of healthy fiber to the glass. Tyrode solution was then replaced with DMEM, 10% FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin (complete medium). Fibers were cultured in complete medium for 24-48 h before confocal microscopy imaging analysis. Isolated fibers were viable in these conditions for at least 10 days. The localization of GFP fusion proteins was determined at a Bio-Rad confocal microscope.

Assessment of transfected fibers

GFP expression was observed on 12 µm thick cryostat sections of TA muscles with a fluorescent microscope (Carl Zeiss, Oberkochen, Germany). The sections were air-dried after sectioning and inspected dry to avoid leaching of GFP to neighboring cells. GFP positive fibers were counted and expressed as percentage of total fibers per section. The localization of GFP fusion proteins was determined at a Bio-Rad confocal microscope. Nuclei were counterstained by Hoechst 33258. Transfected fibers were counted and expressed as percentage of total fibers.

Results

A snap25GFP gene-encoding plasmid was used to assess the conditions of best gene transfer with minimal muscle injury. Preliminary experiments showed that a pair of spatula like electrodes (0.5 cm large) placed at each side of the skeletal muscle gave much higher transfection with less damage than external plates and needle electrodes. We used these modified electrodes for the following experiments. When we applied the conditions of electric pulses used for external plates, we observed massive muscle damage and regeneration. In contrast, we found that five long (20 ms) low electric field (50 V/cm) at a 5-Hz frequency resulted in 79.6% ± 3.2 (mean ± SD) of transfected adult fibers (Figure 1A, B, C) and in a drastic reduction in muscle inflammation. Transgene expression was reduced when we reduced the number of pulses at 3 and 1 to 66% ± 20 and 40% ± 6.8 respectively (Figure 1A). However higher number of pulses increased

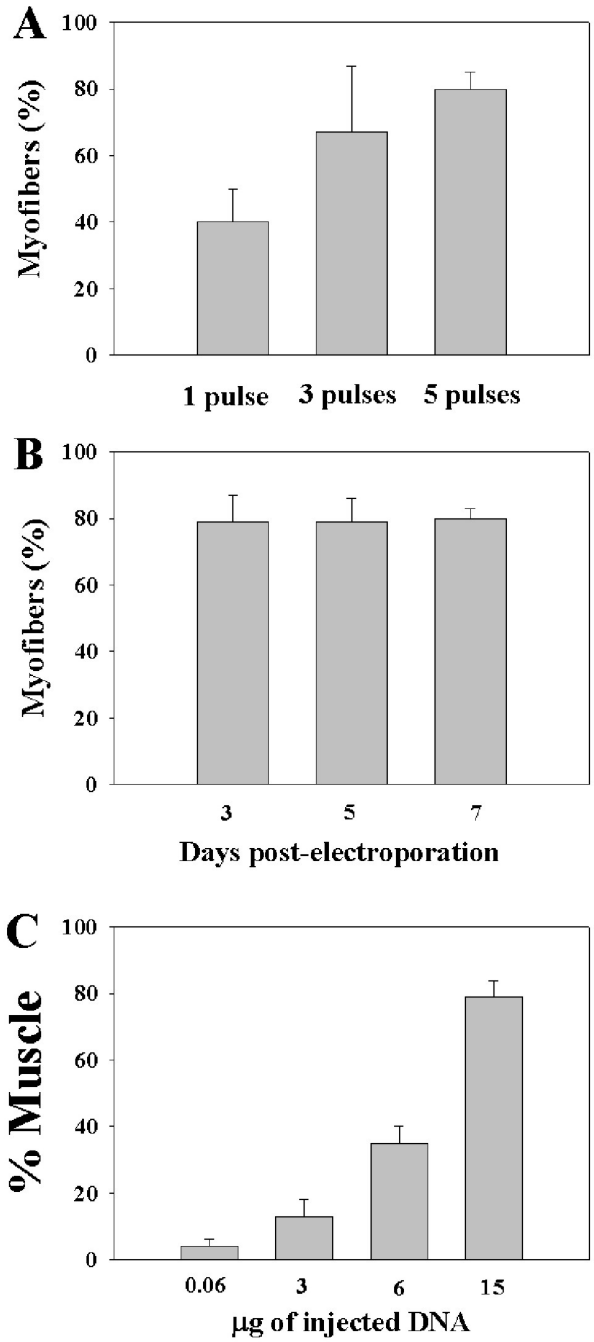

Figure 1. Quantification of Snap-GFP transfected fibers expressed as percentage of the total muscle fiber (mean ± SD).

muscle injury but not gene transfer. A time course of expression was studied at 3, 5, 7 days post-electroporation which resulted in 78.5% ± 8.7, 78.6% ± 7.7, 79.6% ± 3.2 of positive GFP myofibers respectively. Note the consistent gene transfection efficiency and the low variability indicated by the low SD (Figure 1B).

Intramuscular gene electrotransfer increased as a function of the amount of DNA injected remaining high with 15 µg (79% ± 6) and decreasing progressively with 6 µg (27.3% ± 6) and 3 µg (13% ± 8.6) but it was always greater than DNA injected without electroporation that gave a 0.6% ± 0.6 positive fibers per muscle (Figure 1C). Moreover tissue morphology was preserved and only adult myofibers were transfected since regenerating fibers were minimal and untransfected and the inflammatory response was transient.

Next we studied the subcellular distribution of transfected genes using a cytochrome c-GFP and snap25GFP constructs. Confocal microscopy of transfected fibers showed that protein products were localized in the expected subcellular organelles and proteins were expressed along the entire fiber (data not shown). This is an important tool for signal transduction studies and for analyzing organelle functions.

Discussion

Our results show that electric field could be applied for gene transfer into adult skeletal muscle for *in vivo* applications. Indeed, a high number of muscle fiber is successfully transduced using snap25GFP construct and obtaining results similar to viral-mediated gene transfer. We have chosen to quantify reporter gene expression by manual counting of transfected fibers because this type of measurement indicated the topography and extent of transfection, the distribution of the vectors, the presence or absence of an inflammatory reaction and the subcellular localization of the transgene products. Indeed for treatment of muscle disease by gene replacement it is essential to ensure the survival of muscle fibers while maximizing the number of transfected fibers and minimizing inflammatory response. The electrotransfer conditions that we applied did not trigger extended muscle damage as shown by the low number of centronucleated fibers and by the transitory cellular infiltration. Surprising we detected high percentage of transfected fiber even 3-4 weeks after electroporation in mice that were not immunosoppressed by therapy. The absence of muscle damage coupled with an efficient plasmid transfer into adult fibers didn't activate an immune response against constructs at least during our period of observations. Previous report of high efficiency of transfection has also noted the association of muscle damage with efficient gene transfer suggesting a concomitant involvement of transfected myoblast in gene transfer. We obtain higher level of gene transfer when muscle was preserved by damage, suggesting that inflammation and regeneration hinder rather than facilitating a good gene transfer. The reason of the low muscle injury is probably due to the voltage that we applied. In comparison to previous published methods, this protocol permits good electrodes positioning at each side of the muscle allowing the application of a homogeneous low voltage field across the muscle. This reduction in voltage associated with the improved gene transfer leads to a substantial reduction in damage to the muscle.

In conclusion this approach gives the possibility to transfect a high number of adult myofiber in a percentage similar to viral-mediated gene transfer. The applications in gene therapy, in cell physiology, in interfering with

transgene expression in mice, in co-transfecting different constructs are obvious and potentially of broad interest.

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