In vivo plasmid DNA electroporation resulted in transfection of satellite cells and lasting transgene expression in regenerated muscle fibers ☆

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Abstract

In vivo plasmid DNA electroporation resulted in elevated and lasting transgene expression in skeletal muscles. But the nature of the cells that contributed to sustained gene expression remains unknown. We followed the fate of plasmid DNA delivered with electroporation and systematically investigated the time course and location of transgene expression in muscle tissues both with GFP and luciferase. Furthermore, satellite cell activation after electroporation was confirmed by RT-PCR and immunohistochemistry analysis. The activated satellite cells were shown to be able to uptake the injected plasmid DNA and express transgene products as regenerated myocytes. We found that cells with longer gene expression durations were mostly regenerated muscle fibers. In contrast, expression in pre-existing muscle fibers was rather transient. We also presented in this study that immune response to transgene products might hamper the lasting gene expression. Based on these observations, we proposed that the underlying mechanism for prolonged transgene expression in the muscles after electroporation is related to the activation and transfection of myogenic satellite cells which subsequently developed into regenerated muscle fibers.
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Transfection of skeletal muscle fibers in situ for vaccination or production of therapeutic proteins in gene therapy has been shown to have great potentials [1–5]. Direct injection of plasmid DNA solution intramuscularly resulted in noticeable transgene expression in muscles which could last for months [6,7]. Although the gene expression levels were rather low and variable [8,9], the long duration of foreign gene expression without much gene integration was considerably remarkable [10,11]. Different delivery methods had been tried to increase the efficiency of gene transfer in muscles [12,13]. Most significantly, dramatic improvements were found by applying in vivo electroporation after the plasmid DNA injection [14–19]. It was reported that the electroporation treatment may increase reporter gene expression by more than 100-fold and the expression could last for 3–9 months [4,17]. The technique therefore becomes very attractive considering the many possible applications when a significant amount of transgene products can be expressed by the abundant and metabolically active muscle tissues in vivo for local or systemic application [20].

The enhanced effect for gene transfer with electroporation in muscles was suggested to be due to the tissue disruption and cell membrane permeation caused by the electric pulses of electroporation [21–23]. The membrane poration effect of the electric pulses as well as the electrophoretic movements of plasmid DNA were both thought to contribute to the enhanced DNA entry into muscle fibers [23,24]. However, there were also a few other studies that suggested

the possible existence of a receptor-mediated DNA uptake mechanism [25]. But neither of these proposed mechanisms offered a definitive explanation about why in vivo transfection of muscle fibers resulted in a much longer transgene expression duration compared to other tissue types when there was not an active process of gene integration [10,11]. Transgene expression after in vivo somatic gene transfer using nonviral vectors usually would only last for days, but in muscles it could be months. Considering that the plasmids in muscle fibers were found to mostly remain extrachromosomal, and integration into host DNA, if occurred at all, was very limited [26], some studies suggested that lasting transgene expression in muscles might be due to the non-dividing and multi-nuclei nature of the muscle fibers [10,16], but no detailed evidence was shown.

In this study, we systematically investigated the time course of transgene expression in skeletal muscles and found that transgene expression in most pre-existing muscle fibers (cells that have survived injection and electroporation without undergoing necrosis) was transient for about 10 days. Longer and sustained transgene expressions (2–3 weeks post-in vivo DNA electroporation treatment) existed mainly in regenerated muscle fibers. After DNA injection and the electroporation treatment, substantial amount of plasmid DNA was seen to accumulate in myocyte associated satellite cells which were activated due to the damages to muscle fibers. These activated satellite cells eventually developed into regenerated muscle fibers and continued to express transgene for more than 3 weeks. Based on these observations, we therefore proposed a hypothesis in addition to current gene uptake and gene expression mechanisms in muscles after electroporation-mediated intramuscular gene delivery.

Methods

Plasmids and animals. The plasmid gWIZ-GFP (pGFP) encoding for Green Fluorescence Protein and the plasmid pLUC encoding firefly luciferase were both purchased from Gene Therapy Systems (San Diego, CA). They were multiplied in the *Escherichia coli* DH5α bacterial strain and purified using Qiagen Mega Kits (Chatsworth, CA). All other reagents were of analytical grade. Female 6- to 8-weeks-old Balb/c mice and SD rats (100–150 g body weight) were purchased from Animal Center of Fudan University (Shanghai, China). All animal experiment protocols used in this study were designed and performed abiding by the regulations of SJTU and the Chinese government.

In vivo electroporation of DNA. Intramuscular injection (i.m.) of plasmid DNA followed by electroporation (DNA+EP) was performed according to a commonly used protocol. Briefly, the mice were anesthetized by intraperitoneal injection of chloral hydrate at a dose of 10 μl/g of body weight. After complete sedation, each mouse, bilaterally, received 5 μg DNA in 50 μl PBS in one tibialis anterior (TA) muscles using a 27-gauge needle. Immediately after the injection, electroporation with six electric pulses was applied through a pair of silver electrodes spaced 3 mm apart covering the i.m. injection site. The electric pulses were 50 ms in duration and 1 s apart at a voltage of 60 V, i.e., 200 V/cm.

Luciferase expression assays. To quantify the luciferase expression in muscles, mice that had been treated with DNA (pLUC)+EP were sacrificed at specific time points and each TA muscle was surgically removed and homogenized in 1 ml lysis buffer (Promega, Madison) to yield protein

extracts separately (5 mice, 10 TA muscles). Luciferase activities of the protein extracts were determined using a luciferase assay kit (Promega). Samples were counted for 20 s on a Turner Designs Luminometer (Turener Designs, Sunnyvale, CA). Mice received i.m. plasmid DNA injection only were used as control.

GFP expression study and histological analysis. At specific time points, mice that had been treated with DNA (pGFP)+EP were sacrificed and the TA muscles were removed, embedded in OCT, and snap-frozen in isopentane cooled with liquid nitrogen. Serial cross-sections of 7 μm thickness were cut throughout the muscle at −20°C. One out of each 10 sections was selected for examination and representative images were reported. Usually, the samples were examined under a Leica DME fluorescent microscope (Argon light source, filter 488 nm). For samples that require stronger excitation, a confocal microscope (Carl Zeiss) equipped with laser light (488 nm) was used. The number of GFP positive muscle fibers on cross-section was counted under a fluorescent microscope. At least eight sections from four different TA muscles were evaluated at each time point.

For histological analysis, one out of each 10 sections was stained with H&E and examined. The sections near the electrode track and in the peripheral were selected and compared in histological analysis.

Immunohistochemistry and immunofluorescent staining. Serial cross-sections of 7 μm thickness were cut throughout the TA muscles at −20 °C. MyoD protein expressions in rat muscle sections with or without electroporation treatment were examined by immunohistochemistry staining. SD rats were used in the experiment because only anti-rat MyoD mAb were available to us. One day after treatment, sections of TA muscles were fixed in 4 °C acetone and washed with PBS. They were then incubated with 1% hydrogen peroxide to quench endogenous tissue peroxidase and incubated with 1.5% normal rat serum for 30 min to reduce non-specific binding. The sections were then stained with mouse anti-rat MyoD mAb (BD Biosciences) followed by incubation with biotinylated-secondary Ab (goat anti-mouse IgGs) and AB enzyme (Santa Cruz). Sections were developed with chromagen AEC (Biomeda, Forster City) and counterstained with hematoxylin (Sigma).

To determine the differentiation of myogenic cells in muscles, antibody against myogenin (M-255) (Santa Cruz, CA), a rabbit polyclonal antibody, was used. Immunohistochemistry staining was done on TA muscle frozen sections derived from mice 5 days post-in vivo GFP plasmid electroporation. Staining procedure was similar to that previously described. Briefly, sections were fixed in 10% neutral buffered formalin and incubated with 5% normal goat serum to reduce non-specific binding. The sections were then stained with myogenin followed by incubation with rhodamine-conjugated goat-anti-rabbit IgG(H+L) (Santa Cruz, CA). Sections were counterstained with hematoxylin (Sigma) and viewed under a confocal microscope.

Some tissue sections designated for GFP examinations were also stained with rat anti-mouse CD4 (L3T4) mAb (PharMingen, San Diego, CA) or rat anti-mouse CD8α (Ly-2) mAb (PharMingen, San Diego, CA) to identify CD4$^+$ and CD8$^+$ T cells. Sections were incubated with biotinylated-secondary antibody (goat anti-rat IgGs) plus AB enzyme after first antibody application, developed using chromagen AEC, and counterstained with hematoxylin.

Tissue distribution and cellular uptake of plasmid DNA. The pGFP plasmids were labeled with Rhodamine-PNA (Gene Therapy System) which specifically bound to a specifically inserted sequence in the plasmid. 20 μg of labeled plasmids in 50 μl solution was injected to each TA muscle followed by electroporation. After 2 h, mice or rats were sacrificed and TA muscles were removed for frozen sections. Mice sections were directly used to detect the distribution of plasmid DNA. DAPI was used to stain cell nuclear. In some experiments, rat TA muscle sections were stained with mouse anti-rat MyoD mAb (BD Biosciences) followed by incubation with FITC conjugated-secondary antibody (goat anti-mouse IgGs) (BD Biosciences) to identify satellite cells. Sections were examined using a confocal microscope.

RT-PCR. Total RNAs were exacted from TA muscle tissue using a commercial total RNA isolation kit (Sangon, Shanghai, China). The RNA concentration and purity were examined using OD$_{260/280}$ measurements and gel electrophoresis. For MyoD mRNA identification, 2 μg of total