Bacterial β-galactosidase and human dystrophin genes are expressed in mouse skeletal muscle fibers after ballistic transfection

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Abstract Ballistic transfection, based on cell and tissue bombardment by the tungsten and gold microparticles covered with the gene DNA, was used for the delivery of a bacterial β-galactosidase and a full-length cDNA copy of the human dystrophin genes into mouse skeletal muscles. CMV-lacZ, SV40-lacZ, LTR-lacZneo and full-length cDNA dystrophin (pDMD-1, approximately 16 kb) in eukaryotic expression vector pJ OMEGA driven by mouse leukaemia virus promotor (pMLVDy) were used throughout the studies. *Musculus glutaeus superficialis* of C57BL/6J and *quadriceps femoris* of mdx male mice were opened surgically under anesthesia and bombarded by means of the gene-gun technique originally developed by us. Different mixtures of gold and tungsten particles at ratios of 4:1, 1:1, 1:4 were applied. X-gal assay revealed marked β-gal activity, both in total muscles and whole muscle fibers on histological sections, up to three months after transfection. The most intensive staining was observed after SV40-lacZ delivery. No staining was detected with LTR-lacZneo DNA as well as in untreated muscles. The higher tungsten particle concentration in the bombardment mixture correlated with more intense X-gal staining. At the gold/tungsten ratio of 1:4 the microparticles penetrated the *musculus glutaeus superficialis* and transfected the underlying *musculus glutaeus medius* as well. Immuno-cytochemical assay for human dystrophin revealed dystrophin positive myofibers (DPM) in the bombarded area up to two months after transfection. The proportion of DMP varied from 2.5% on day 17 up two 5% on day 60 after bombardment compared to only 0.5% in the control mdx mice. These results suggest the applicability of particle bombardment for gene delivery into muscle fibers.

Key words: Ballistic transfection; In vivo, bacterial β-galactosidase and human dystrophin genes; Mouse skeletal muscle

1. Introduction

Introduction of exogenous gene constructions into skeletal muscle tissues is regarded as a possible approach towards gene therapy of a number of inherited disorders including common monogenic diseases such as Duchenne muscular dystrophy (DMD) [1–4]. Several attempts to introduce foreign genes into muscle tissues have been undertaken during the last decade. After injection of bacterial β-galactosidase and some other reporter genes into mouse muscle tissue their expression was demonstrated up to two months [5].

It has been repeatedly shown that human dystrophin gene when introduced into skeletal muscle of mdx mice (biological model of DMD) retains the ability for efficient expression [6–10].

In the most of these studies direct DNA injection via standard syringe needles was applied. We decided to use the ballistic (gene-gun) approach based on the target object bombardment by heavy metal (tungsten or gold) microparticles coated with DNA [11–13]. The approach has proved itself to be highly efficient for animal cell and tissues transfection in situ [13–19], including muscle transfection [18,19]. One to three days after bombardment the activities of the introduced CAT [18] and luciferase [19] genes were detected in mouse muscles. However, the methods of detection used in these investigations (study of organ extracts) did not allow elucidation whether gene expression was confined to the muscle fibers themselves or could be attributed to some other cell types such as connective tissue.

The main goal of the present study was to estimate the capacity of ballistic method to deliver reporter genes as well as therapeutic dystrophin gene into muscle fibers in vivo. To our knowledge, the present study describes the first attempt to exploit microparticle bombardment technique for the introduction of dystrophin gene into skeletal muscle fibers of mdx mice.

2. Materials and methods

2.1. Manipulation with animals

C57BL/6J and mdx dystrophin deficient male mice, two–eight weeks old, were used throughout the studies. *Musculi glutaeus superficialis* and *quadriceps femoris* were opened surgically under avertin anesthesia and bombarded through the fascia by means of gene-gun technique as indicated below. After the bombardment, the skin was stitched up and the mice were returned to the animal house. 100% of animals survived the procedure. The animals were killed from 24 h to three months later, the bombarded area of the muscle was taken for X-gal or dystrophin assays [10].

In parallel experiments, both water solutions and calcium phosphate precipitates of plasmid DNA were injected into muscles in the

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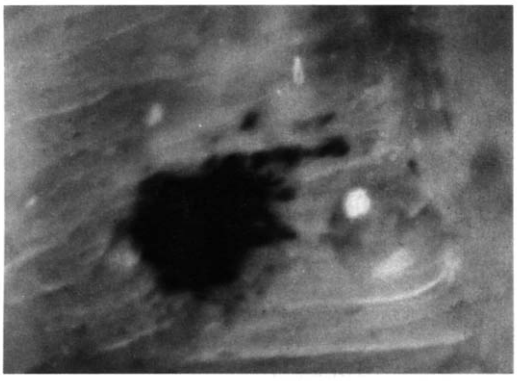

Fig. 1. X-gal positive blue stained area in gene-bombarded C57BL/6J mouse *musculus glutaeus superficialis* three months after transfection with SV40-lacZ DNA. Total muscle preparation. Approximate size of the stained area is 1.5×1 mm.

same amount as in the gene-gun experiments. Untreated muscles and those after injection of β-galactosidase protein water solution were used as controls.

2.2. Transfection

The shooting technology was mainly similar to that described previously [15,16]. The distance between the end of the barrel and the tissue surface was about 10 cm. To reduce the contamination of tissues with toxic products of the explosive charge, two diaphragms with the center apertures of increasing diameter have been placed between the barrel and the bombarded tissue. The first diaphragm contains a metal grid 1.5 mm×1.5 mm mesh in size and the second one has a grid 0.14 mm×0.14 mm mesh in size. The first grid is placed near the end of the barrel to decrease cell damage by dispersing particles of a destroyed macroprojectile. The second grid located as close as possible to the bombarded tissues disintegrates any conglomerates of metal microparticles formed in the course of DNA precipitation. Both grids substantially facilitate the efficiency of the transfection.

The golden (1 μm in diameter) and tungsten (1–4 μm in diameter) microparticles alone, or in a mixture in different ratios (4:1, 1:1, 1:4 respectively) were used. 20–30 μg of circular plasmid DNA was taken per shot.

CMV-lacZ, SV40-lacZ, LTR-lacZneo and full-length cDNA dystrophin (pDMD-1, approximately 16 kb) in eukaryotic expression vector p14 driven by mouse leukemia virus promotor MLV, pMLVDy [10] were used throughout the studies.

Plasmid DNA was prepared by the standard lysozyme/alkaline lysis followed by CsCl gradient centrifugation. Calcium phosphate precipitation [12] was used to coat the particles with plasmid DNA. 20–30 μl of a DNA solution (1 mg/ml) added to 10–15 mg of microparticles were taken per one shot.

2.3. Determination of transgene activities

The presence of bacterial β-galactosidase in muscle fibers and tissues was detected using standard X-gal assay both in blocks of total muscle or on cryosections.

For dystrophin immunostaining, muscle cryosections were treated with a protein G purified rabbit polyclonal antibody (P6) raised against the C-terminus of dystrophin and subsequently with FITZ or horseradish peroxidase labelled sheep anti-rabbit IgG (secondary antibody).

3. Results and discussion

Three days after transfection, β-gal assay of gene-bombarded muscle revealed typical blue stained areas (up to 3×4 mm). The expression was detected up to three months after transfection (Fig. 1, the later periods were not studied). The staining was most conspicuous after application of SV40-lacZ construction delivery. No specific staining was detected in experiments with LTR-lacZneo DNA and in untreated muscles.

After direct intramuscular injection of pCMV-LacZ DNA, marked but rather weak X-gal staining was observed. Direct injections of bacterial β-galactosidase protein resulted in the appearance of blue stained areas.

In all these experiments, only superficial muscles contained the stained areas, most likely due to the limited particle penetration into the muscle tissue. The investigation carried out on serial cryosections across the bombarded muscles confirmed this assumption. After the bombardment with gold

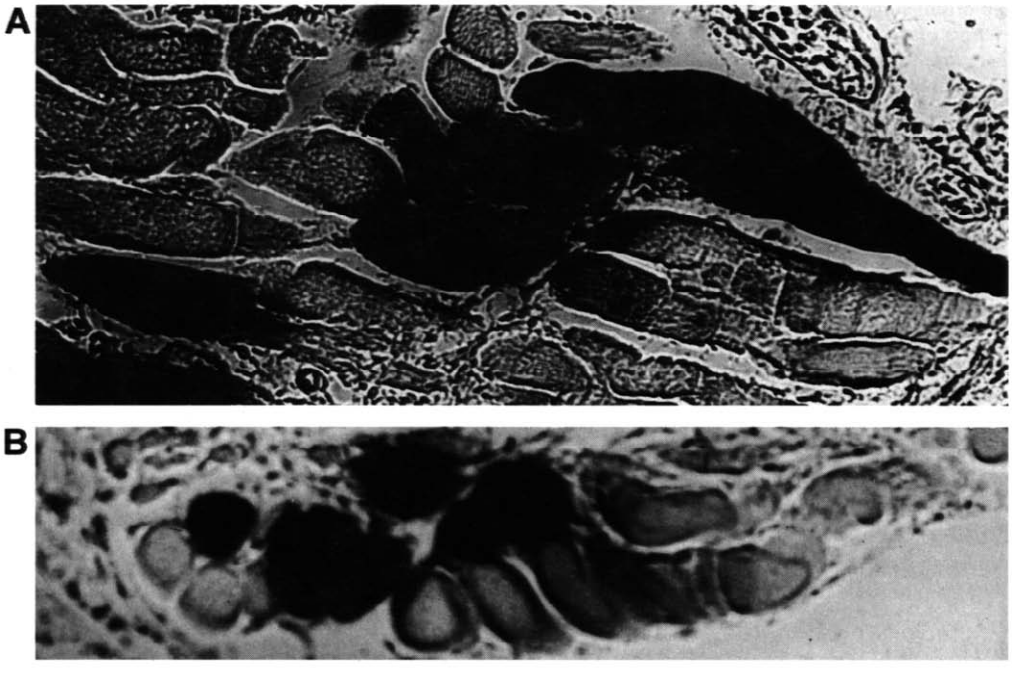

Fig. 2. X-gal positive muscle fibers in *musculus glutaeus medius* of C57BL/6J mouse three days after ballistic introduction of SV40-lacZ DNA. A: Longitudinal section; B: cross-section.

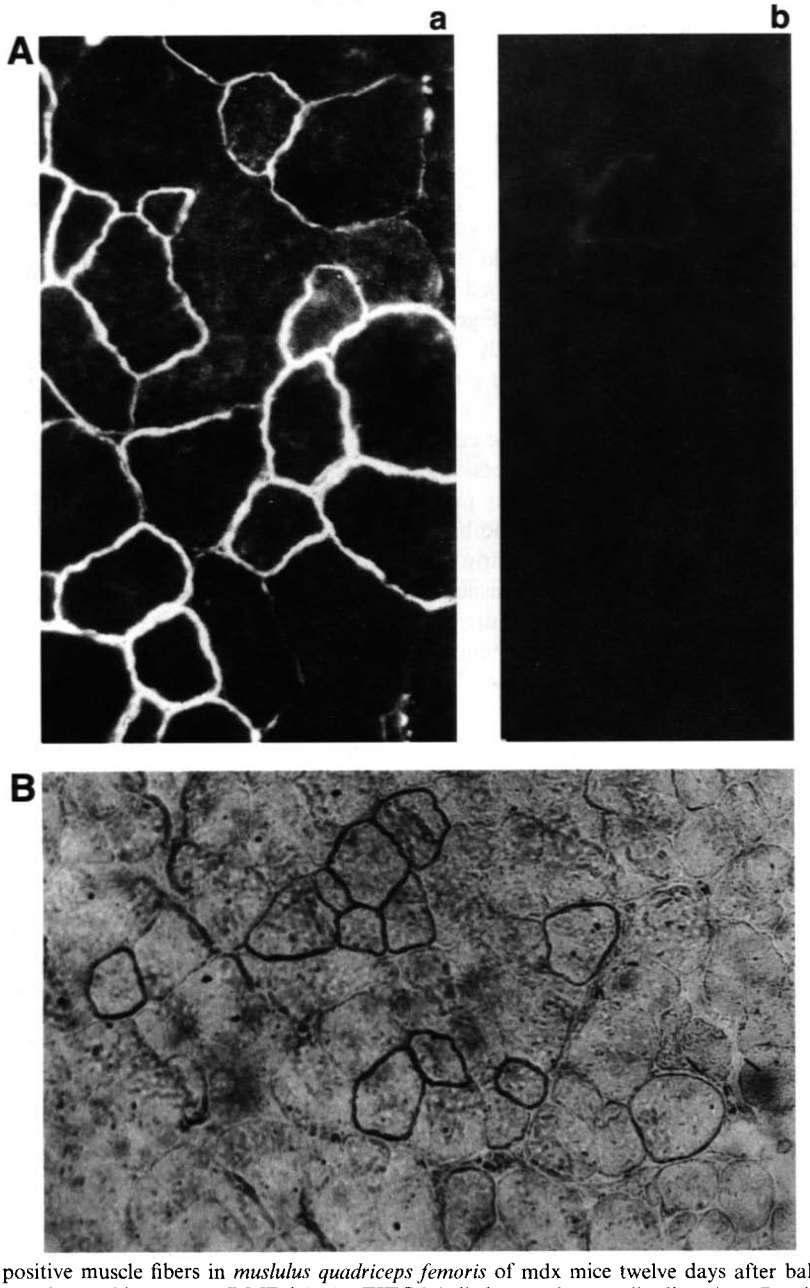

Fig. 3. Clusters of dystrophin positive muscle fibers in *muslulus quadriceps femoris* of mdx mice twelve days after ballistic introduction of a full-length cDNA copy of the human dystrophin genes (pDMDdy). A: FITC-labelled secondary antibodies. A.a: Bombarded muscle (experiment). A.b: Non-bombarded muscle (negative control). B: Horseradish peroxidase secondary antibodies.

particles 1.0–4.0 mm in size the deepness of the particle penetration did not exceed 0.5 mm.

To improve the bombardment conditions we used the mixture of gold and tungsten microparticles. Experiments showed that the higher was tungsten particle concentration, the thicker was the X-gal stained muscle layer. At the gold/tungsten ratio of 1:4, the microparticles penetrated through the *musculus glutaeus superficialis* and transfected the underlying *musculus glutaeus medius* as well. Thus, under these conditions the deepness of gene-gun transfections was at least 4 mm.

Further experiments were carried out using these newly developed bombardment conditions.

In the next series of experiments, β-gal expression was studied in cross-sections of the ballistically transfected muscle fibers of *musculi glutaeus superficialis* and *glutaeus medius*. The X-gal assay revealed intense and massive blue staining in whole muscle fibers (Fig. 2). The experiments thus proved that the foreign gene introduced ballistically into mouse muscle is expressed in its fibers.

The technique optimized using the bacterial β-galactosidase gene was then applied in the studies with the human dystrophin gene construction.

Full cDNA copy of a human dystrophin gene, pMSVDy, was introduced into *musculus quadriceps femoris* of mdx mice which were then killed 3–60 days after bombardment. A number of mdx mouse muscle fibers in bombarded area were found to be dystrophin positive. The proportion of dystrophin positive myofibers (DPM) varied from 2.5% on day 17 up to 5% on day 60 after bombardment, compared to only 0.5% in

the control mdx mice of the same age. Clusters comprising 25–30 DMP fibers running at least through 350–400 microns of successive histological sections were of special interest (Fig. 3).

The results obtained clearly demonstrate the applicability of gene-gun technique for efficient transfection of muscle fibers by foreign genes.

Complementary DNA copies of the human dystrophin gene were introduced by particle bombardment into the skeletal muscles of mdx mice and were shown to be expressed in clusters of fibers for at least two months. The area of gene expression after ballistic transfection was significantly increased compared to that after direct injection of naked plasmid into the muscle of mdx mice.

The actual proportion of DPM is still too small to be considered seriously for gene therapy studies. Nonetheless, in combination with some other new sophisticated vectors (adenoviruses, cationic liposomes, complex vectors, etc.) the ballistic transfection might be used not only in gene therapy of muscle genetic diseases but also for heterologous transgene delivery and prolonged expression in this tissue. Further investigations of transfection efficiency of the gene-gun technique combined with other vector systems are now in progress.

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