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Direct gene transfer into mouse diaphragm

Heather L. Davis[a,b,*], Bernard J. Jasmin[b]

[a]*Physiotherapy Program, Faculty of Health Sciences, University of Ottawa, 451 Smyth Road, Ottawa, K1H 8M5, Canada*
[b]*Department of Physiology, Faculty of Medicine, University of Ottawa, Ottawa, K1H 8M5, Canada*

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Direct gene transfer into skeletal muscle is a potential therapeutic strategy for inherited primary myopathies such as Duchenne muscular dystrophy (DMD). In order to affect the life-expectancy of these patients, it will be necessary to carry out gene therapy on the diaphragm. To this end, we report efficient introduction of pure recombinant plasmid DNA into the mouse diaphragm, without causing significant damage. Application of this approach to the diaphragm of the *mdx* mouse will provide information on the potential usefulness of gene therapy for the treatment of DMD patients.

Gene transfer; Muscle; Diaphragm; Muscular dystrophy

1. INTRODUCTION

Many of the primary myopathies are known or thought to be caused by single gene defects, and thus represent potential candidates for gene therapy. The most prevalent primary myopathy is the Duchenne form of muscular dystrophy (DMD), a fatal X-linked recessive disorder affecting 1 in 3,500 males [1]. DMD is characterized by progressive muscular atrophy and degeneration with concomitant loss of function [2]. The genetic defect responsible for this disease is located at Xp21 and prevents the production of dystrophin, a large (427 kDa) structural protein associated with the sarcolemma [3–5]. The muscle pathology consists of persistent cycles of degeneration and regeneration with eventual failure to regenerate leading to the replacement of myofibres by fatty and fibrotic tissue. Essentially all skeletal muscles, including the diaphragm are affected, and death, which occurs in the second or third decade of life, most often results from respiratory insufficiency.

In attempts to develop therapeutic strategies for DMD, direct gene transfer into muscle has been evaluated in animal models. Reporter genes have been successfully introduced into hindlimb muscles of mice using pure plasmid DNA [6–10], adenovirus [6,11–13] or retrovirus [6,14]. Although these results are encouraging, particularly with adenovirus and pure plasmid DNA, the efficiency of transfer is still insufficient to consider human trials. In addition, a major concern has been the potential difficulty to carry out direct gene transfer on the diaphragm and/or intercostal muscles, which would be essential in order to affect the life expectancy of these patients.

*Corresponding author. Fax: (1) (613) 787 6675.

As part of our on-going studies using direct gene transfer to study the physiological regulation of membrane and cytoskeletal proteins in muscle, we investigated the feasibility of introducing new genetic material into the diaphragm. Here we report the development of an approach that successfully allows the uptake and expression of pure recombinant plasmid DNA by a large number of muscle fibres in the mouse diaphragm, without causing significant damage. These findings have implications for the treatment of primary myopathies such as DMD.

2. MATERIALS AND METHODS

2.1. *Expression vectors*

Recombinant plasmid DNA containing *Escherichia coli lacZ* for β-galactosidase (β-gal) as reporter gene driven by the cytomegalovirus (CMV) promoter [15] (pCMV-*lacZ*), as previously reported [16], was prepared by the Qiagen (Chatsworth, CA) mega-prep procedure. The DNA was recovered by ethanol precipitation, then redissolved in water, aliquoted and stored at −80°C until required for injection.

2.2. *In vivo gene transfer into diaphragm*

Gene transfer was carried out on diaphragms of male C57BL/6J mice (Charles River) aged 6–7 weeks (20 21 g), under anesthesia (sodium pentobarbital, 75 mg/kg i.p.). The inferior surface of the left hemi-diaphragm was exposed via a 2 cm incision made inferior to the lateral costal margin. While holding the costal margin such that the peripheral muscular part of the hemi-diaphragm was flattened, the DNA solution (pCMV-*lacZ*, 2 μg/μl; in 0.1 M PBS, pH 7.4) was injected at several sites between the inferior surface of the muscle and the overlying epimysium. With the aid of a surgical microscope, this was carried out using a 1 ml TB syringe and a 30 g needle. The needle was inserted, orifice side away from the muscle, until the entire beveled portion was contained within the fascia. Each hemi-diaphragm received a total volume of 50 μl in 4–8 individual injections placed around the perimeter of the muscle. The contralateral hemi-diaphragm served as an untreated control. Subsequent to DNA injection, abdominal musculature and skin were closed with 5.0 silk and wound clips, respectively.

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2.3. *Histochemistry*

Five days after gene transfer, the entire diaphragm was removed and stained for activity of β-gal either in the whole muscle or on cryostat cross-sections.

For staining of whole muscle, the entire diaphragm, still attached to the inferior ribs and xiphoid process, was removed and pinned onto paraffin in a Petri dish. Staining was carried out with a modification of the method of Sanes et al. [17]. Specimens were fixed for 4–16 h at 4°C in 2% paraformaldehyde, 2 mM $MgCl_2$ and 1.25 mM EDTA in PBS (0.1 M, pH 7.4), rinsed 3 × 30 min in PBS at 4°C and pre-incubated in detergent solution (2 mM $MgCl_2$, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 0.01% sodium desoxycholate and 0.02% Nonidet NP 40 in PBS). Incubation was for 4–8 h at 37°C in detergent solution containing 400 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-Gal). The X-Gal was first dissolved in DMFO as a 4% w/v stock, which was stored at −20°C. Following incubation, muscles were rinsed in PBS and stored in the fixative solution at 4°C.

For cryostat sectioning, individual hemi-diaphragms were dissected from the ribs and wrapped around a piece of liver, then were frozen in isopentane cooled by liquid N_2. The entire muscle was sectioned (12 μm), and every tenth section was collected onto a gelatinized slide and stained for β-gal by a modification of the method of Dannenburg and Suga [18]. Sections were fixed for 15 min at 4°C in 1% glutaraldehyde in PBS, rinsed 3 × 5 min in PBS at 4°C, incubated for 2–6 h at 37°C (PBS containing X-Gal at 400 mg/ml, 1 mM $MgCl_2$, 5 mM potassium ferrocyanide and 5 mM potassium ferricyanide; X-Gal made from stock as above), rinsed 2 × 5 min in PBS, counterstained in alcoholic eosin (1 min in 0.25%), dehydrated through graded alcohols, cleared in xylene and mounted in Permount. Serial sections to those stained for β-gal were also stained by (i) routine haematoxylin and eosin (H&E), to detect the presence of centronucleation, or (ii) immunohistochemical detection of embryonic and neonatal isoforms of myosin heavy chain (MyHC) using the BF-45 antibody of Schiaffino et al. [19].

3. RESULTS AND DISCUSSION

3.1. *Efficient transfer of DNA into muscle fibres of diaphragm*

In contrast to methods we have recently developed for efficient transfer of genetic material directly into mouse hindlimb muscle, in which pure DNA or viral vectors are injected intramuscularly following pre-treatment with hypertonic sucrose [7] or in regenerating muscles [6], we decided upon an alternative approach owing to the thinness of diaphragm muscle. Sub-epimysial injection of plasmid DNA (pCMV-*lacZ*) into mouse diaphragm resulted in uptake and expression of the *lacZ* reporter gene. As shown in Fig. 1a, each injection resulted in staining of several fibres in the vicinity of the injection site. Fibres were frequently stained along their entire length, from the costal margin to the central tendon. Although the DNA was applied to only the inferior surface of the diaphragm, all injections resulted in staining of both superficial and deeper fibres, with several injection sites exhibiting β-gal expressing fibres accross the entire 10–12 cell thickness of the muscle (Figs. 1b, 2a and 2c). Individual injection sites resulted in 41 ± 3 stained muscle fibres ($x \pm$ S.E.M., $n = 8$), which is superior to results obtained with injection of similar quantities of pCMV-*lacZ* into hindlimb mouse muscle. Surprisingly, the present results compare favorably with those obtained with injection of DNA into sucrose pretreated tibialis anterior (TA) muscle, in which a single injection of 4–5 times more DNA resulted in only 36 ± 7 *lacZ*-expressing fibres ($n = 6$) [6]. This may reflect better diffusion of the DNA within the diaphragm, which may occur as a result of a less dense endomysium and/or an assistive action of its rhythmic contractions.

3.2. *Sub-epimysial injection causes minimal damage*

In our previous studies on gene transfer into TA muscle, intramuscular injection of DNA caused damage to a large number of fibres, which underwent a cycle of degeneration and regeneration [7]. Sub-epimysial injection of DNA, however, resulted in little or no damage to muscle fibres in the diaphragm, as evidenced by the paucity of fibres having centrally placed nuclei (Fig. 2a and b) or containing immature (embryonic and neonatal) isoforms of MyHC (Fig. 2c and d). Furthermore, when damaged and/or regenerating fibres were observed, they were usually restricted to the first two layers of muscle cells on the surface of the diaphragm immediately adjacent to the injection site (Fig. 2e–g). An additional concern in these experiments, was the effect of the multiple injection procedure per se on the normal functioning of the diaphragm. During the injections, the rhythm of diaphragmatic contractions was undisturbed. Furthermore, animals recovered quickly post-operatively and did not appear to present any particular signs of discomfort or respiratory distress. Thus, this injection procedure does not seem to have deleterious consequences for the animal.

3.3. *Therapeutic implications*

The results reported here represent the first demonstration of direct gene transfer into diaphragm muscle. Furthermore, they show that sub-epimysial injection of diaphragm is safe and results in efficient gene transfer while causing minimal myofibre damage. Our results thus can be applied to the *mdx* mouse to provide a model with which to investigate the potential usefulness of gene therapy for DMD. The *mdx* mouse [20], which like DMD has a virtual lack of dystrophin, has been limited as an experimental model since its muscles, with the apparent exception of the diaphragm, fail to develop significant pathological changes [21]. We are currently testing the ability of full-length and mini-dystrophin

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Fig. 1. Sub-epimysial injection of plasmid DNA results in uptake and expression of *lacZ* reporter gene in diaphragm muscle fibers. Diaphragm stained for activity of β-gal in the whole muscle (A) or cryostat section (B) are shown. (A) β-gal activity is detected only in the injected hemi-diaphragm and is restricted to injection sites. Note also that the injected side shows staining from the costal margin to the central tendon. (B) Stained fibers are present throughout the thickness of the muscle (see also Fig. 2a and c). Magnifications are × 9 (A) and × 209 (B).


A
B

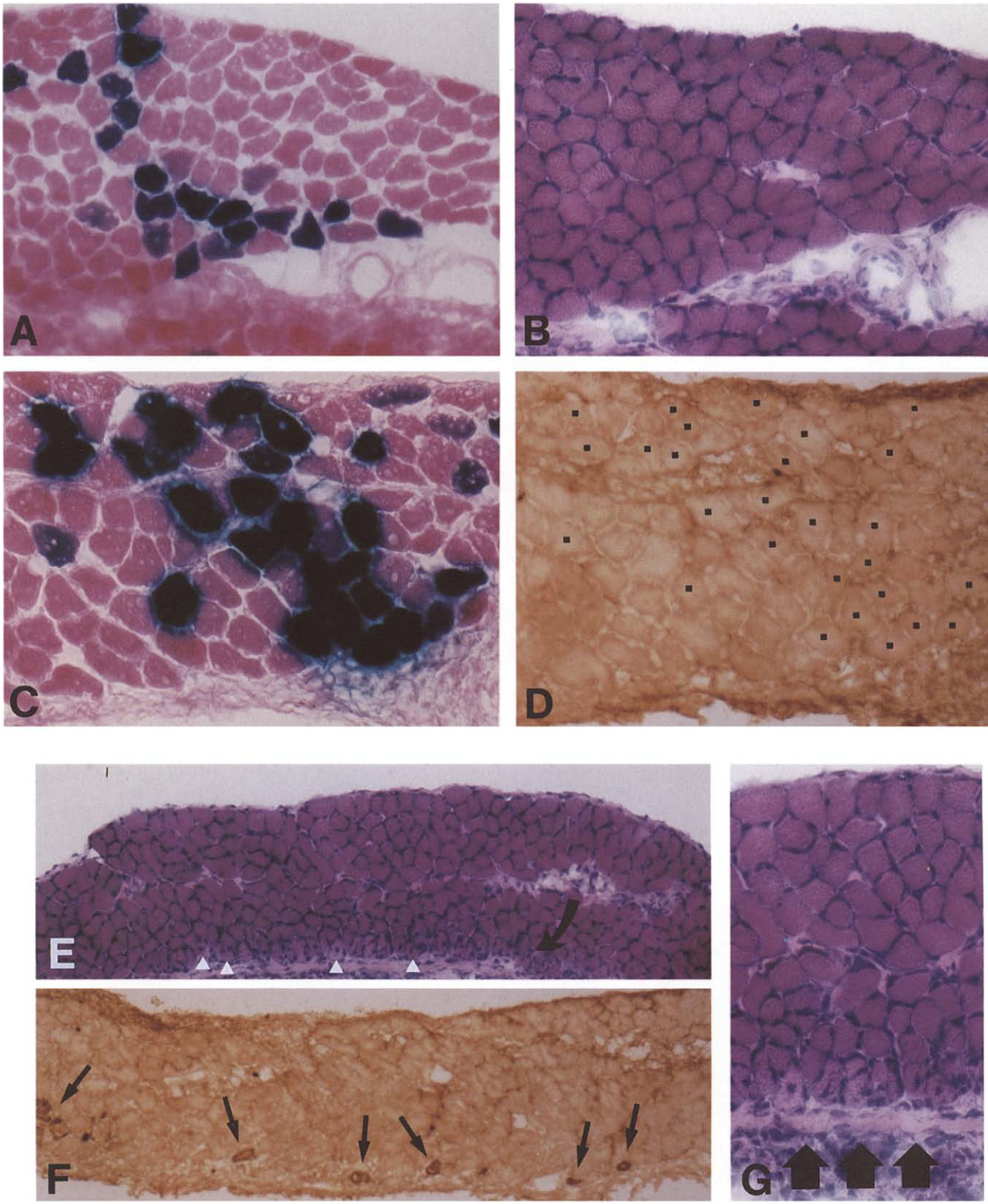

Fig. 2. Sub-epimysial injection causes minimal muscle fiber damage. A/B and C/D represent serial cross-sections of diaphragm muscle injected with *lacZ* reporter gene and 5d later stained for β-gal activity (A,C), H & E (B), or embryonic and neonatal isoforms of MyHC immunoreactivity (D). Note that muscle fibers expressing the reporter gene are found throughout the thickness of the diaphragm (A,C) and that these fibers do not show signs of damage (B,D). Some muscle fibers display signs of damage as evidenced by centrally located myonuclei (see arrows in E and G). Damaged fibers were few and restricted to the most superficial part of the muscle adjacent to the site of injection. (F) shows a muscle cross-section area stained for the presence of embryonic and neonatal isoforms of MyHC; few immunoreactive fibers are present (arrows). Magnifications are $\times 209$ (A,B,C,D,G) and $\times 105$ (E,F).

expression vectors to prevent or reverse pathological changes in the *mdx* diaphragm. Based on the recent finding that expression of human dystrophin can correct the myopathic phenotype in transgenic *mdx* mice [22,23], gene therapy using dystrophin expression constructs appears to be a promising avenue for improving function in muscles of DMD patients.

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