Ultrasound Increases Plasmid-Mediated Gene Transfer to Dystrophic Muscles without Collateral Damage

Gawiyou Danialou,[1] Alain S. Comtois,[1] Roy W. R. Dudley,[1] Josephine Nalbantoglu,[2] Renald Gilbert,[2] George Karpati,[2] David H. Jones,[3] and Basil J. Petrof[1,2,*]

[1]*Respiratory Division, Department of Medicine, McGill University Health Centre, and Meakins-Christie Laboratories, McGill University, Montreal, Quebec, Canada H3A 1A1*
[2]*Neuromuscular Research Group, Montreal Neurological Institute, McGill University, Montreal, Quebec, Canada H3A 2B4*
[3]*Department of Exercise Science, Concordia University, Montreal, Quebec, Canada H3G 1M8*

[*]*To whom correspondence and reprint requests should be addressed. Fax: (514) 843-1695. E-mail: basil.petrof@mcgill.ca.*

Studies have shown that ultrasound, used either alone or in combination with microbubble contrast agents, can increase cell membrane permeability to plasmid DNA. Because ultrasound is a non-painful and well-established tool in clinical medicine, its potential to enhance DNA uptake into the muscles of patients with muscular dystrophy is conceptually attractive. Therefore, we evaluated the ability of ultrasound pulses (1 MHz; 1.5 W/cm^2) to increase exogenous (LacZ) gene expression in normal wild-type and dystrophic *Dmd*$^{mdx/mdx}$ mice after plasmid DNA injection into muscle. We also ascertained whether co-injection of lipid-encapsulated perfluoropropane microbubbles (Definity) or pretreatment with hyaluronidase could further increase the level of gene transfer to ultrasound-treated muscles. The use of ultrasound did not increase transfection efficiency in normal mice. In contrast, dystrophic mice demonstrated an increase in the number of transfected fibers (threefold) as well as the amount of LacZ protein (22-fold) after ultrasound exposure, provided that Definity was also co-injected with the DNA. Pretreatment of muscles with hyaluronidase before ultrasound exposure was not effective in augmenting the level of gene transfer. Under the optimal conditions for dystrophic muscle transfection (ultrasound + Definity), there was no associated increase in muscle damage. Hence ultrasound may provide a safe and effective method for enhancing gene transfer to dystrophic muscles, thereby increasing the prospects for therapeutic application of naked DNA in muscular dystrophy patients.

Key Words: sonoporation, naked DNA, myofiber transfection, ultrasound contrast agents, microbubbles, *mdx* mouse, Duchenne muscular dystrophy

INTRODUCTION

Muscular dystrophies are a heterogeneous group of diseases that may be associated not only with substantial morbidity, but also with early mortality due to respiratory and cardiac muscle involvement. Advances in molecular biology over the past several years have permitted the identification of several defective genes underlying these disorders. Chief among these are defects of the gene encoding dystrophin, a subsarcolemmal cytoskeletal protein which has an important role in maintaining the physical integrity of the muscle cell surface membrane [1]. Lack of the dystrophin protein leads to Duchenne muscular dystrophy (DMD), a relatively common (1 in 3500 live male births) and ultimately fatal X-linked form of muscular dystrophy. At the present time, treatment for the disease is largely supportive. Given the lack of effective treatment options, there has been much interest in the potential applicability of gene therapy to DMD patients.

Since the original report by Wolff and colleagues [2] that exogenous gene expression can be achieved *in vivo* after direct intramuscular injection of plasmid DNA, there has been great interest in the potential applicability of this procedure to the treatment of muscular dystrophy. Plasmid DNA injection has been shown to produce gene transfer not only in appendicular muscles, but also in muscles that are critical for survival such as the heart [3] and diaphragm [4,5]. However, a major limitation to this approach has been the fact that transfection efficiency is generally very low [2,6].

Several studies have shown that ultrasound, used either alone or in combination with ultrasound contrast agents, can increase cell membrane permeability to macromolecules such as plasmid DNA [7–10]. This phenomenon has

1525-0016/02 $35.00

doi:10.1006/mthe.2002.0723, available online at http://www.idealibrary.com on IDEAL

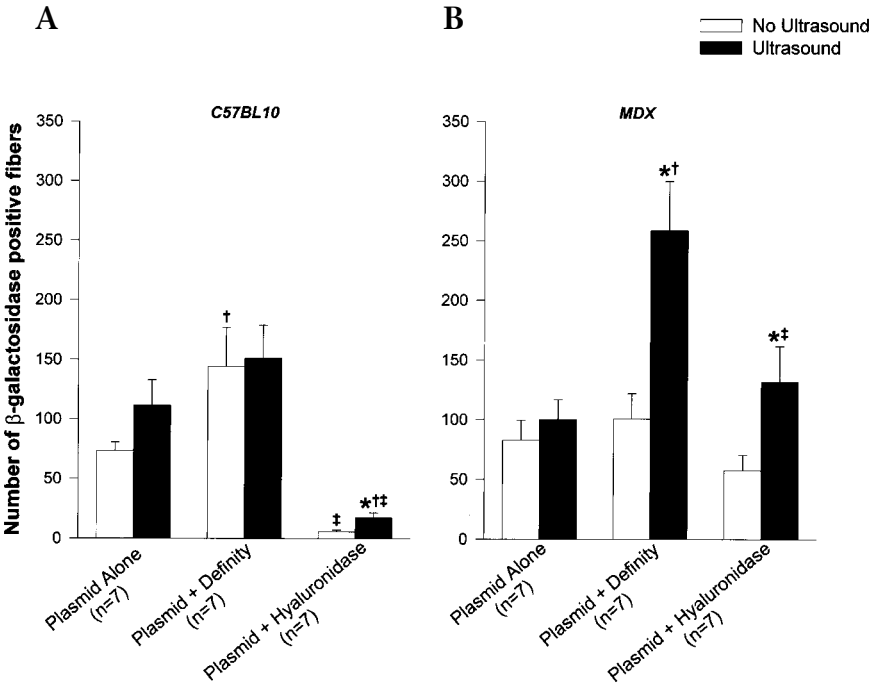

FIG. 1. Quantitation of β-gal-expressing fibers after LacZ plasmid DNA administration to the tibialis anterior muscles of wild-type (A) and dystrophic (B) mice. The total number of β-gal-expressing fibers on the entire muscle cross section was determined 5 days after DNA injection in the absence (open bars) or presence (filled bars) of ultrasound. Data are mean values ± SE. *$P < 0.05$, within group comparison to no ultrasound. +$P < 0.05$, across group comparison to plasmid alone. ‡$P < 0.05$, across group comparison to plasmid + Definity.

been referred to as sonoporation [7]. Most sonoporation studies have been carried out on cultured cells [7–10] or tumors *in vivo* [11–14], and very little is known about the effects of ultrasound on direct gene transfer to skeletal muscle *in vivo*. In addition, to our knowledge there has been no previous investigation of whether ultrasound is able to enhance plasmid-mediated gene transfer to dystrophic muscles.

Therefore, the global objective of our study was to investigate the potential usefulness of ultrasound as a method for improving the efficiency of plasmid-mediated gene transfer to skeletal muscle in general, and to dystrophic muscles in particular. In this study we used the *Dmd*$^{mdx/mdx}$ mouse (hereafter referred to simply as *mdx*), an animal model of DMD lacking dystrophin due to a point mutation in the gene [15], in order to evaluate the response to ultrasound in dystrophic muscles. Our specific aims were as follows: 1) to determine effects of ultrasound application on gene transfer efficiency after plasmid DNA injection into normal and dystrophic muscles *in vivo*; 2) to evaluate the ability of adjuvant compounds, such as a lipid-based microbubble ultrasound contrast agent or the extracellular matrix-degrading enzyme hyaluronidase, to enhance ultrasound-mediated effects on gene transfer; and 3) to ascertain whether the use of ultrasound to augment plasmid-mediated gene transfer is associated with muscle damage in either normal or dystrophic muscles.

RESULTS

To evaluate the effects of ultrasound on skeletal muscle transfection efficiency, tibialis anterior muscles of wild-type and dystrophic mice were injected with 25 μg of plasmid and euthanized 5 days later. The effects of ultrasound on expression of the *lacZ* gene product β-galactosidase (β-gal) were determined under three different experimental conditions: 1) use of plasmid alone; 2) use of plasmid + Definity; and 3) use of plasmid + hyaluronidase pretreatment. Figure 1 shows the mean number of muscle fibers expressing β-gal in all groups, whereas Fig. 2 shows levels of β-gal protein in the corresponding whole muscle homogenates.

In wild-type C57BL10 mice (Fig. 1A), application of ultrasound had no significant effect on the number of β-gal-expressing muscle fibers after injection of plasmid alone. Similarly, use of Definity as an adjuvant agent did not significantly increase the efficacy of ultrasound treatment in wild-type mice. On the other hand, ultrasound did increase the number of transfected fibers in hyaluronidase pretreated muscles, although the overall level of transfection was significantly lower than in muscles not subjected to hyaluronidase. In addition, it is interesting to note that use of Definity on its own (that is, without ultrasound) was associated with a statistically significant increase in the number of transfected fibers in wild-type mice.

A very different picture emerged in dystrophic muscle (Fig. 1B). Neither Definity nor ultrasound on their own had any significant effects on the number of transfected fibers. However, when Definity and ultrasound were applied in combination, there was a substantial increase (approximately threefold) in the number of transfected myofibers compared with plasmid alone without ultrasound. In addition, a significant effect of ultrasound on transfection efficiency was also seen in muscles subjected to hyaluronidase pretreatment. However, as was the case in wild-type mice, the absolute number of transfected myofibers found after hyaluronidase pretreatment in dystrophic mice was not superior to that obtained in muscles injected with plasmid alone.

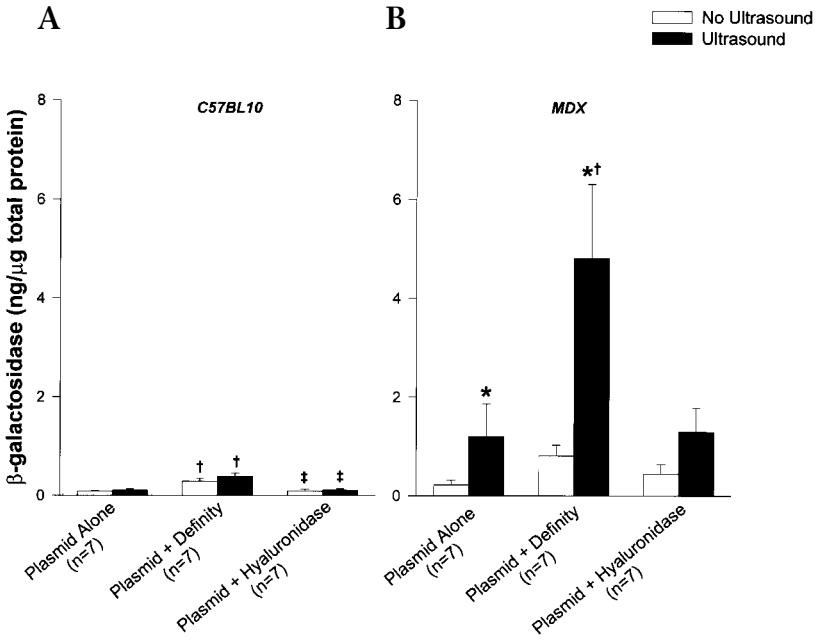

FIG. 2. Level of β-gal expression in muscle homogenates from the tibialis anterior muscles of wild-type (A) and dystrophic (B) mice. β-Gal activity was determined 5 days after DNA injection in the absence (open bars) or presence (filled bars) of ultrasound. Data are mean values ± SE. *$P < 0.05$, within group comparison to no ultrasound. +$P < 0.05$, across group comparison to plasmid alone. ‡$P < 0.05$, across group comparison to plasmid + Definity.

Measurements of β-gal protein by chemiluminescence in whole muscle homogenates were generally consistent with the above morphometric data. Application of ultrasound to muscles of normal wild-type mice had no significant effects on β-gal protein expression under any of the conditions studied (Fig. 2A). However, a small but significant effect of Definity by itself was observed once again in C57BL10 (but not dystrophic) mice.

In dystrophic mice, ultrasound significantly increased the magnitude of β-gal expression in the plasmid alone as well as the plasmid + Definity groups, with a similar trend also being found in the hyaluronidase pretreatment group (Fig. 2B). The highest level of β-gal protein expression was found in the plasmid + Definity group treated with ultrasound, which increased approximately 22-fold compared with plasmid alone without ultrasound. Thus, the relative increase in β-gal protein levels after ultrasound exceeded the corresponding changes in transfected fiber number, suggesting that the level of β-gal expression per fiber was also increased. This is supported by the representative examples of β-gal staining provided in Fig. 3, which show that not only the number, but also the intensity, of β-gal staining was increased in ultrasound-treated muscles receiving plasmid + Definity.

Because the highest transfection efficiency was obtained in muscles receiving plasmid + Definity, potential tissue damage related to the application of ultrasound was evaluated under these conditions and compared with plasmid alone (Fig. 4). As expected, the baseline level of muscle damage was higher in dystrophic mice than in wild-type animals. However, use of ultrasound did not increase the magnitude of muscle damage in wild-type or dystrophic muscles injected with either plasmid alone or plasmid + Definity.

DISCUSSION

Here we have explored the potential of muscle-directed ultrasound to improve transfection efficiency of intramuscularly injected plasmid DNA in dystrophic as well as normal muscles. The principal findings of our study were as follows: 1) significant ultrasound-mediated enhancement of gene transfer by plasmid DNA was found in dystrophic muscles, but not in the muscles of normal wild-type mice; 2) the beneficial effects of ultrasound on gene transfer in dystrophic muscles were largely dependent upon the concomitant use of a microbubble ultrasound contrast agent (Definity), whereas the combination of ultrasound and pretreatment of muscles with the extracellular matrix-degrading enzyme hyaluronidase was not effective; and 3) the use of ultrasound (either with or without Definity) to increase gene transfer efficiency was not associated with increased muscle damage.

Possible Mechanisms Underlying Ultrasound Effects

Although there is some evidence supporting a role for receptor-mediated uptake of naked DNA by cells [16], the actual mechanism of plasmid uptake by myofibers after direct injection *in vivo* remains uncertain. Moreover, physical methods which are not expected to involve a receptor-mediated mechanism can modify transfection efficiency. For example, electroporation greatly increases *in vivo* transfection efficiency when applied shortly after direct intramuscular injection of naked DNA [17–19]. Unfortunately, this procedure is also associated with a substantial degree of muscle damage [18,19].

In the present study, we hypothesized that application of ultrasound could have beneficial effects on *in vivo* transfection by plasmid DNA without inducing significant muscle damage. Several studies have shown that ultrasound can be used to enhance cellular transfection, both *in vitro* [7–10] and *in vivo* [11–14,20]. In principal, such ultrasound effects could be exerted at a number of different levels including plasmid DNA entry into the cell, intracellular trafficking, and enhanced transcriptional as well as translational activity. However, it is likely that at least one

doi:10.1006/mthe.2002.0723, available online at http://www.idealibrary.com on IDEAL

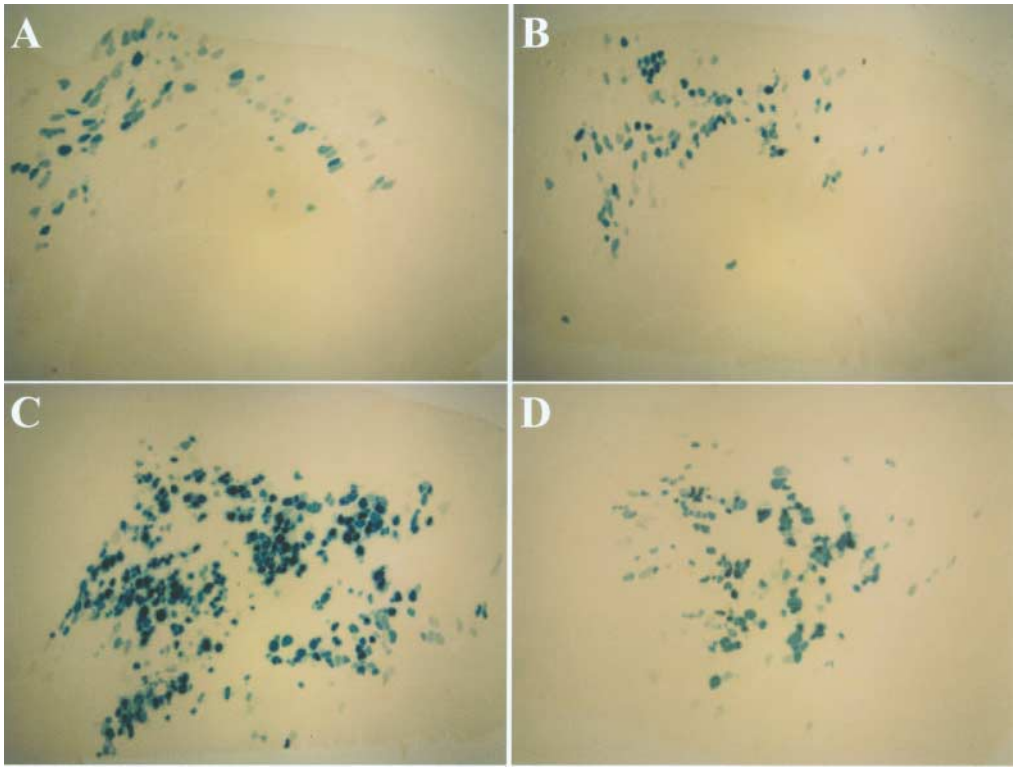

FIG. 3. Representative photomicrographs of histochemical staining for β-gal in the dystrophic mouse tibialis anterior muscle for the following experimental groups: (A) plasmid alone without ultrasound; (B) plasmid alone with ultrasound; (C) plasmid + Definity with ultrasound; and (D) plasmid + hyaluronidase with ultrasound. Note that in addition to an increase in the number of β-gal expressing fibers, the intensity of staining per fiber is increased in (C).

mechanism is via transient pore formation within the cell membrane, a process termed sonoporation [7]. Indeed, such pore formation [20,21], as well as entry of high molecular weight dextrans [7] into cells after application of ultrasound, has been well documented.

Acoustic cavitation, which is defined by the presence of gas microbubbles that have been set into motion by exposure to an acoustic field, seems to be the primary mechanism underlying increases in cell membrane permeability and DNA delivery into cells after exposure to ultrasound [7,10,22,23]. The microbubbles may be formed intrinsically by the advancing acoustic waves (particularly in tissues with high levels of dissolved gases) or may be supplied exogenously as in the present study (that is, Definity). Cell membranes can be permeabilized by several cavitation-related phenomena. These include mechanical forces exerted by oscillating bubbles or microstreaming; liquid jets or shock waves following unstable or transient cavitation, in which the bubbles grow and then collapse violently; and the release of free radicals during the final stages of bubble growth and collapse [10,23,24].

An important finding in the present investigation was the differential susceptibility of normal and dystrophin-deficient muscles to ultrasound-mediated effects on transfection efficiency. This may reflect a greater susceptibility of dystrophin-deficient myofibers to the effects of acoustic cavitation on the muscle cell membrane. Indeed, it is well established that muscle cells lacking dystrophin have altered mechanical properties. This is manifested by an increased vulnerability to hypo-osmotic shock [25] and reduced membrane stiffness *in vitro* [26], as well as an increased susceptibility to sarcolemmal disruptions triggered by forceful muscle contractions *in vivo* [27,28]. Muscle cells lacking dystrophin are also reportedly more vulnerable to oxidative stress [29]. Therefore, to the extent that acoustic cavitation can create both mechanical and oxidative stresses [24], it is perhaps not surprising that muscles lacking dystrophin respond differently from normal muscles to the application of ultrasound pulses. Moreover, additional factors such as the presence of regenerating myofibers within dystrophic muscles may also have a role in the greater transfection efficiency found in dystrophin-deficient muscles exposed to ultrasound.

Enhancement of Ultrasound Effects by Definity

Injection of microbubble-containing ultrasound contrast agents can greatly increase cavitation effects on cells during ultrasound exposure [7,23]. Such contrast agents are approved for human use and consist of different types of stabilized microbubbles surrounded by protein, sugar, or lipid shells [23,30,31]. In the present study, we used Definity, an injectable form of lipid-encapsulated perfluoropropane microbubbles. The use of a lipid-based compound in this study was considered as a potential advantage, as it might allow the effects of liposomal and ultrasound-mediated transfection to be combined. Such has been shown to be the

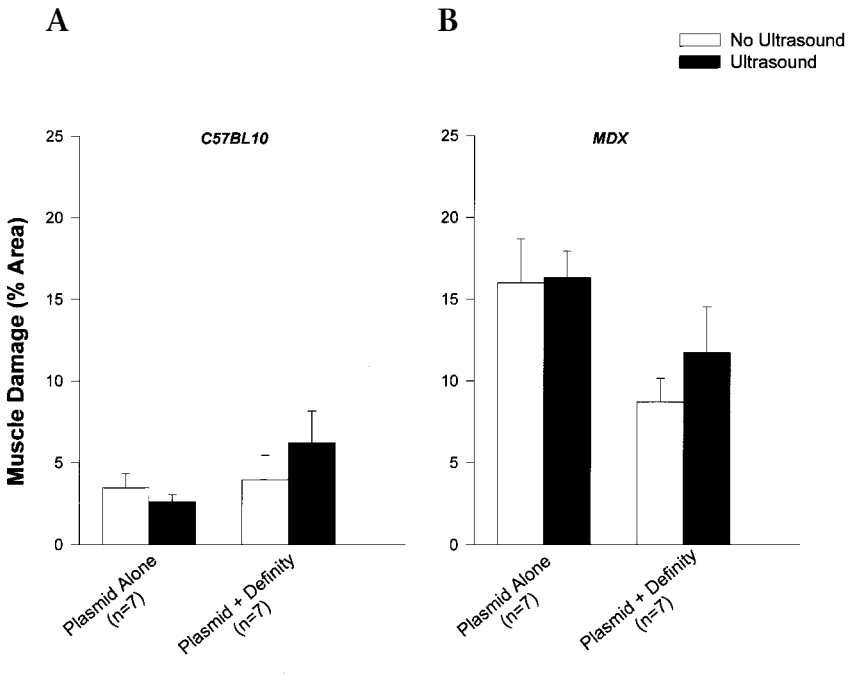

FIG. 4. Level of muscle damage and inflammation in wild-type (A) and dystrophic (B) mouse muscles at 5 days after DNA injection in the absence (open bars) or presence (filled bars) of ultrasound. There was no significant effect of ultrasound (with or without concomitant use of Definity) on the level of muscle damage in either mouse strain. Data are mean values ± SE.

case in several different cell types *in vitro* [22,32], as well as in mouse tumor models studied *in vivo* [12].

We found that while ultrasound did not augment transfection efficiency in normal muscles, use of Definity on its own produced a significant increase in both β-gal protein levels and the number of transfected fibers. On the other hand, use of Definity in isolation had no significant effects in dystrophic mice, but was essential for allowing ultrasound-induced enhancement of transfection in this group. These results differ somewhat from those of Taniyama *et al*. [20], who recently reported an increased transfection efficiency of normal rat muscles exposed to ultrasound after plasmid DNA injection. The different responses of normal muscles to ultrasound observed in our study are likely related to methodological factors such as the use of different ultrasound parameters and microbubble contrast agents, as well as the different animal species used. However, similar to our findings in dystrophic mice, these authors found that use of an albumin-encapsulated microbubble contrast agent (Optison) was needed to achieve significant effects [20].

Lack of Increased Muscle Damage after Ultrasound

Injection of plasmid DNA into muscle is known to cause mild inflammation, which has been linked to the presence of proinflammatory unmethylated CpG motifs within prokaryotic DNA [33,34]. Consistent with previous reports by other investigators [35], we also observed a mild degree of interstitial inflammation 5 days after plasmid DNA injection. This occurred in the absence of ultrasound exposure and amounted to approximately 5% of cross-sectional area in normal muscles. However, it is important to note that ultrasound itself was not associated with any further increase in muscle damage or inflammation.

Maximizing transfection efficiency depends on increasing membrane porosity while avoiding irreversible cellular damage. It is known that breaches of the cell surface membrane of muscle fibers can be resealed and thus allow necrosis to be avoided [36,37]. In addition, to the extent that the influx of inflammatory cells associated with muscle damage boosts the host immune response against transgene-encoded proteins [38], less damage may also translate into lower immunogenicity. Hence the absence of increased damage with ultrasound in our study not only increases the clinical acceptability of this intervention, but it may also allow for more sustained transgene expression over time.

In contrast, application of *in vivo* electroporation produces muscle injury and inflammation, with one-third to one-half of the muscle cross-sectional area showing damage in some studies [18,19]. The propensity of *in vivo* electroporation to cause muscle damage likely accounts for the observation that transfection efficiency is approximately 50% lower in *mdx* mice than in normal wild-type mice [39]. This suggests a greater vulnerability to electroporation-induced damage in dystrophin-deficient fibers, which would help explain the reduced level of transgene expression [39]. The results of our study suggest that ultrasound may be better able to achieve the optimal balance between membrane pore formation and resealing in dystrophic muscles.

Implications for Gene Therapy of Muscular Dystrophy

An important and still unresolved issue for gene therapy of DMD is the most appropriate choice of vector. Thus far, most research in this area has involved viral vectors. Although the latter are highly efficient gene transfer vehicles in muscle, a significant disadvantage of virus-based systems is the host immune response elicited by these vectors [38,40,41]. This has raised important safety concerns, and also triggers neutralizing antibodies against viral proteins that can prevent effective readministration [42,43]. Stimulation of cytotoxic T-cell responses may also occur, and this leads to the transduced muscle cell population

doi:10.1006/mthe.2002.0723, available online at http://www.idealibrary.com on IDEAL

being destroyed in the absence of immunosuppressive therapy [40–42]. In addition, not all viral vectors (for example, adeno-associated virus) are able to accommodate the full-length dystrophin gene. An alternative approach is to use a nonviral vector such as plasmid DNA, which is less toxic, cheaper, easier to prepare, and able to accommodate the full-length dystrophin cDNA. However, the major drawback of the naked DNA approach up to now has been the very low gene transfer efficiency compared with viral vectors.

The use of ultrasound as an adjuvant measure to enhance plasmid DNA delivery has a number of advantageous features, which should increase the overall prospects for therapeutic application of naked DNA in muscle. In contrast to electroporation, ultrasound is a non-painful and well-established tool in clinical medicine. Furthermore, ultrasound is currently used to treat musculoskeletal injuries in humans [44], and animal studies suggest the potential for positive effects on muscle repair processes [45,46]. Whereas electroporation is less effective in dystrophic muscles [39], precisely the opposite pattern was observed with ultrasound (that is, a greater efficacy of ultrasound-induced transfection in *mdx* than in normal mouse muscles). Equally important, there was no histological evidence of increased damage in dystrophic muscles subjected to ultrasound. The non-invasive nature of ultrasound and the absence of neutralizing antibodies against plasmid DNA vectors also raise the possibility that treatments could be easily repeated on a relatively frequent basis, thereby permitting selected muscles to be progressively "loaded" with therapeutic DNA in a step-wise fashion. Lastly, ultrasound-mediated destruction of intravascularly injected microbubble contrast agents has been used to induce microvessel breaches that are large enough to permit extravasation of macromolecules, including gene transfer vectors [47,48]. Therefore, ultrasound could be a powerful adjunct to intravascular as well as intramuscular delivery of plasmid DNA to dystrophic muscles. Further studies will be required to investigate the full therapeutic potential of ultrasound-based approaches to gene delivery in the setting of muscular dystrophy.

MATERIALS AND METHODS

Preparation of plasmid DNA. Plasmid DNA was produced according to standard methods [5] and purified to remove contamination with endotoxin. The plasmid contained the LacZ cDNA, which was driven by a chicken β-actin promoter/cytomegalovirus enhancer construct [49]. All experimental groups were injected with a single pooled sample of plasmid DNA in order to eliminate any potential variation caused by the use of different DNA preparations.

Animal procedures. Dystrophic *mdx* and normal wild-type (C57BL10) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The mice were provided with food and water ad libitum. Before intramuscular injection of plasmid DNA, the mice were anesthetized with ketamine (130 mg/kg) and xylazine (20 mg/kg). Mice were euthanized by anesthetic overdose. All experimental protocols were approved by the institutional animal care and ethics committee.

Animals were studied at 8–10 weeks of age. Tibialis anterior (TA) muscles of both hindlimbs were initially injected with plasmid DNA (25 μg). One TA muscle was exposed to ultrasound treatment immediately after plasmid administration, whereas the opposite TA muscle served as a within-animal control. A commercially available sonicator (Ultramax Excel, Excel Tech Ltd, Oakville, Ontario) was used to transcutaneously deliver ultrasound pulses of 3 ms duration at 1 MHz frequency and 1.5 W/cm^2 intensity, with an inter-pulse interval of 7 ms (30% duty cycle). These settings were selected to promote gene transfer while minimizing tissue injury related to thermal effects of ultrasound [45]. Ultrasound gel (EcoLotion, Eco-Med Pharmaceuticals Inc., Mississauga, Ontario) was used as a coupling agent, and the ultrasound head was held in a stationary position over the TA muscle during the entire treatment period of 2 minutes.

Pre-injection of microbubble ultrasound contrast agents has been shown to increase the permeabilizing effects of ultrasound on cells [20,23,30,31]. In addition, pretreatment of skeletal muscles with the extracellular matrix-degrading enzyme hyaluronidase increases the efficiency of plasmid-mediated gene transfer by *in vivo* electroporation [19]. Therefore, we also evaluated the ability of such adjuvant measures to increase gene transfer efficiency after ultrasound treatment of normal and dystrophic muscles. The ultrasound contrast agent Definity (Bristol-Myers Squibb Canada, Inc., Saint-Laurent, Quebec), a formulation of lipid-encapsulated perfluoropropane microbubbles, was mixed with the plasmid DNA (16.5 μl of Definity in a total injectate volume of 50 μl) by vortexing for 30 seconds immediately before intramuscular injection. Before mixing of the DNA with Definity, the latter was rapidly agitated using a mechanical vial shaker supplied by the manufacturer. Because the physical properties of Definity can be altered by passage through a small diameter needle, a 20-gauge needle was used (as suggested by the manufacturer) for most plasmid injections. However, for hyaluronidase pretreatment experiments (25 μl of 400 U/ml injected two hours before plasmid DNA delivery [19]), a 27-gauge needle was used for plasmid DNA injection of the muscles.

Detection of transgene expression by chemiluminescence. Quantitation of the *lacZ* gene product, β-gal, was performed on tissue homogenates using a commercially available kit (Galacto-Light, Tropix, Inc., Bedford, MA). Each muscle sample was blotted dry and weighed before freezing in liquid nitrogen. It was then pulverized into a powder, suspended in 200 μl of lysis solution and homogenized. The homogenate was assayed for β-gal activity by chemiluminescence according to the manufacturer's instructions. A standard curve was carried out using commercially available β-gal protein (Boehringer Mannheim, Laval, Quebec). Values for β-gal expression were normalized to total protein within the sample.

Morphometric quantification of gene transfer efficiency and muscle damage. Muscle sections (10 μm) were fixed in 1.5% glutaraldehyde in PBS for 3 minutes and then rinsed with PBS at room temperature. Sections were stained overnight for β-gal activity by incubation at 37°C with 400 μg/ml X-gal, 1 mM $MgCl_2$, 5 mM potassium ferrocyanide, and 5 mM ferricyanide in PBS. Microscopically visualized sections were photographed using a digital camera and the image was stored on a computer. Analysis of the number of individual muscle fibers was performed by manual tag using a commercial software package (Image-Pro Plus, Media Cybernetics, Silver Springs, MD). Fiber counts were performed from cross-sections exhibiting the maximal number of blue fibers, irrespective of the intensity of blue staining.

To quantify muscle damage, muscle sections were stained with hematoxylin and eosin. The muscle section with the greatest amount of inflammatory cell infiltration was chosen for muscle damage evaluation. This was done by determining the area fraction (expressed as a percentage) of damage, which was calculated by dividing the sum of all areas containing inflammatory cells and myofiber degeneration by the total area of the tissue section [42].

Statistical analysis. All data were analyzed with a statistics software package (SigmaStat, Jandel Scientific, San Rafel, CA). Differences between ultrasound-treated and untreated groups were determined using Student's two-tailed *t*-test for independent samples. Differences within ultrasound-treated or untreated groups were tested by one-way ANOVA, with post-hoc application of the Tukey procedure where appropriate. Statistical significance was defined as $P < 0.05$.

ACKNOWLEDGMENTS

This work was supported by grants from the Muscular Dystrophy Association, Inc. (USA), the Canadian Institutes of Health Research, and the Fonds de la recherche en sante du Quebec. G.D. was supported by a fellowship from the Foundation for Gene and Cell Therapy. R.W.R.D. received a studentship award from the Canadian Lung Association.

RECEIVED FOR PUBLICATION SEPTEMBER 4;
ACCEPTED SEPTEMBER 25, 2002.

REFERENCES

1. Hoffman, E. P., Brown, R. H. J., and Kunkel, L. M. (1987). Dystrophin: the protein product of the Duchenne muscular dystrophy locus. *Cell* **51**: 919–928.
2. Wolff, J. A., *et al.* (1990). Direct gene transfer into mouse muscle in vivo. *Science* **247**: 1465–1468.
3. Lin, H., Parmacek, M. S., Morle, G., Bolling, S., and Leiden, J. M. (1990). Expression of recombinant genes in myocardium in vivo after direct injection of DNA. *Circulation* **82**: 2217–2221.
4. Davis, H. L., and Jasmin, B. J. (1993). Direct gene transfer into mouse diaphragm. *FEBS Lett.* **333**: 146–150.
5. Petrof, B. J., Acsadi, G., Bourdon, J., Matusiewicz, N., and Yang, L. (1996). Phenotypic and immunologic factors affecting plasmid-mediated *in vivo* gene transfer to rat diaphragm. *Am. J. Physiol.* **270**: L1023–L1030.
6. Acsadi, G., *et al.* (1991). Human dystrophin expression in mdx mice after intramuscular injection of DNA constructs. *Nature* **352**: 815–818.
7. Bao, S., Thrall, B. D., and Miller, D. L. (1997). Transfection of a reporter plasmid into cultured cells by sonoporation *in vitro*. *Ultrasound Med. Biol.* **23**: 953–959.
8. Kim, H. J., Greenleaf, J. F., Kinnick, R. R., Bronk, J. T., and Bolander, M. E. (1996). Ultrasound-mediated transfection of mammalian cells. *Hum. Gene Ther.* **7**: 1339–1346.
9. Tata, D. B., Dunn, F., and Tindall, D. J. (1997). Selective clinical ultrasound signals mediate differential gene transfer and expression in two human prostate cancer cell lines: LnCap and PC-3. *Biochem. Biophys. Res. Commun.* **234**: 64–67.
10. Wyber, J. A., Andrews, J., and D'Emanuele, A. (1997). The use of sonication for the efficient delivery of plasmid DNA into cells. *Pharm. Res.* **14**: 750–756.
11. Bao, S., Thrall, B. D., Gies, R. A., and Miller, D. L. (1998). *In vivo* transfection of melanoma cells by lithotripter shock waves. *Cancer Res.* **58**: 219–221.
12. Anwer, K., *et al.* (2000). Ultrasound enhancement of cationic lipid-mediated gene transfer to primary tumors following systemic administration. *Gene Ther.* **7**: 1833–1839.
13. Huber, P. E., and Pfisterer, P. (2000). *In vitro* and *in vivo* transfection of plasmid DNA in the Dunning prostate tumor R3327-AT1 is enhanced by focused ultrasound. *Gene Ther.* **7**: 1516–1525.
14. Manome, Y., Nakamura, M., Ohno, T., and Furuhata, H. (2000). Ultrasound facilitates transduction of naked plasmid DNA into colon carcinoma cells *in vitro* and *in vivo*. *Hum. Gene Ther.* **11**: 1521–1528.
15. Sicinski, P., *et al.* (1989). The molecular basis of muscular dystrophy in the mdx mouse: a point mutation. *Science* **244**: 1578–1580.
16. Budker, V., *et al.* (2000). Hypothesis: naked plasmid DNA is taken up by cells *in vivo* by a receptor-mediated process. *J. Gene Med.* **2**: 76–88.
17. Mir, L. M., *et al.* (1999). High-efficiency gene transfer into skeletal muscle mediated by electric pulses. *Proc. Natl. Acad. Sci. USA* **96**: 4262–4267.
18. Mathiesen, I. (1999). Electropermeabilization of skeletal muscle enhances gene transfer *in vivo*. *Gene Ther.* **6**: 508–514.
19. McMahon, J. M., Signori, E., Wells, K. E., Fazio, V. M., and Wells, D. J. (2001). Optimisation of electrotransfer of plasmid into skeletal muscle by pretreatment with hyaluronidase - increased expression with reduced muscle damage. *Gene Ther.* **8**: 1264–1270.
20. Taniyama, Y., *et al.* (2002). Development of safe and efficient novel nonviral gene transfer using ultrasound: enhancement of transfection efficiency of naked plasmid DNA in skeletal muscle. *Gene Ther.* **9**: 372–380.
21. Tachibana, K., Uchida, T., Ogawa, K., Yamashita, N., and Tamura, K. (1999). Induction of cell-membrane porosity by ultrasound. *Lancet* **353**: 1409.
22. Koch, S., Pohl, P., Cobet, U., and Rainov, N. G. (2000). Ultrasound enhancement of liposome-mediated cell transfection is caused by cavitation effects. *Ultrasound Med. Biol.* **26**: 897–903.
23. Greenleaf, W. J., Bolander, M. E., Sarkar, G., Goldring, M. B., and Greenleaf, J. F. (1998). Artificial cavitation nuclei significantly enhance acoustically induced cell transfection. *Ultrasound Med. Biol.* **24**: 587–595.
24. Miller, M. W., Miller, D. L., and Brayman, A. A. (1996). A review of *in vitro* bioeffects of inertial ultrasonic cavitation from a mechanistic perspective. *Ultrasound Med. Biol.* **22**: 1131–1154.
25. Menke, A., and Jockusch, H. (1991). Decreased osmotic stability of dystrophin-less muscle cells from the mdx mouse. *Nature* **349**: 69–71.
26. Pasternak, C., Wong, S., and Elson, E. L. (1995). Mechanical function of dystrophin in muscle cells. *J. Cell Biol.* **128**: 355–361.
27. Petrof, B. J., Shrager, J. B., Stedman, H. H., Kelly, A. M., and Sweeney, H. L. (1993). Dystrophin protects the sarcolemma from stresses developed during muscle contraction. *Proc. Natl. Acad. Sci. USA* **90**: 3710–3714.
28. Danialou, G., *et al.* (2001). Dystrophin-deficient cardiomyocytes are abnormally vulnerable to mechanical stress-induced contractile failure and injury. *FASEB J.* **15**: 1655–1657.
29. Rando, T. A., Disatnik, M.-H., Yu, Y., and Franco, A. (1998). Muscle cells from mdx mice have an increased susceptibility to oxidative stress. *Neuromuscul. Disord.* **8**: 14–21.
30. Dalecki, D., *et al.* (1997). The influence of contrast agents on hemorrhage produced by lithotripter fields. *Ultrasound Med. Biol.* **23**: 1435–1439.
31. Miller, D. L., and Gies, R. A. (2000). The influence of ultrasound frequency and gas-body composition on the contrast agent-mediated enhancement of vascular bioeffects in mouse intestine. *Ultrasound Med. Biol.* **26**: 307–313.
32. Lawrie, A., *et al.* (1999). Ultrasound enhances reporter gene expression after transfection of vascular cells *in vitro*. *Circulation* **99**: 2617–2620.
33. Jakob, T., Walker, P. S., Krieg, A. M., Udey, M. C., and Vogel, J. C. (1998). Activation of cutaneous dendritic cells by CpG-containing oligodeoxynucleotides: a role for dendritic cells in the augmentation of Th1 responses by immunostimulatory DNA. *J. Immunol.* **161**: 3042–3049.
34. Hemmi, H., *et al.* (2000). A Toll-like receptor recognizes bacterial DNA. *Nature* **408**: 740–745.
35. McMahon, J. M., Wells, K. E., Bamfo, J. E., Cartwright, M. A., and Wells, D. J. (1998). Inflammatory responses following direct injection of plasmid DNA into skeletal muscle. *Gene Ther.* **5**: 1283–1290.
36. Carpenter, S., and Karpati, G. (1989). Segmental necrosis and its demarcation in experimental micropuncture injury of skeletal muscle fibers. *J. Neuropathol. Exp. Neurol.* **48**: 154–170.
37. McNeil, P. L., and Khakee, R. (1992). Disruptions of muscle fiber plasma membranes. Role in exercise-induced damage. *Am. J. Pathol.* **140**: 1097–1109.
38. Cordier, L., *et al.* (2001). Muscle-specific promoters may be necessary for adenoassociated virus-mediated gene transfer in the treatment of muscular dystrophies. *Hum. Gene Ther.* **12**: 205–215.
39. Vilquin, J. T., *et al.* (2001). Electrotransfer of naked DNA in the skeletal muscles of animal models of muscular dystrophies. *Gene Ther.* **8**: 1097–1107.
40. Acsadi, G., *et al.* (1996). Dystrophin expression in muscles of mdx mice after adenovirus-mediated *in vivo* gene transfer. *Hum. Gene Ther.* **7**: 129–140.
41. Petrof, B. J., *et al.* (1996). Impairment of force generation after adenovirus-mediated gene transfer to muscle is alleviated by adenoviral gene inactivation and host $CD8^+$ T cell deficiency. *Hum. Gene Ther.* **7**: 1813–1826.
42. Guibinga, G.-H., *et al.* (1998). Combinatorial blockade of calcineurin and CD28 signaling facilitates primary and secondary therapeutic gene transfer by adenovirus vectors in dystrophic (mdx) mouse muscles. *J. Virol.* **72**: 4601–4609.
43. Chirmule, N., *et al.* (2000). Humoral immunity to adeno-associated virus type 2 vectors following administration to murine and nonhuman primate muscle. *J. Virol.* **74**: 2420–2425.
44. Dyson, M. (1985). Therapeutic applications of ultrasound. In: *Biological Effects of Ultrasound (Clinics in Diagnostic Ultrasound)* (W. L. Nyberg and M. Ziskin, Eds.), pp. 121–133. Churchill Livingstone, Inc., New York, NY.
45. Rantanen, J., Thorsson, O., Wollmer, P., Hurme, T., and Kalimo, H. (1999). Effects of therapeutic ultrasound on the regeneration of skeletal myofibers after experimental muscle injury. *Am. J. Sports Med.* **27**: 54–59.
46. Karnes, J. L., and Burton, H. W. (2002). Continuous therapeutic ultrasound accelerates repair of contraction-induced skeletal muscle damage in rats. *Arch. Phys. Med. Rehabil.* **83**: 1–4.
47. Song, J., *et al.* (2002). Influence of injection site, microvascular pressure and ultrasound variables on microbubble-mediated delivery of microspheres to muscle. *J. Am. Coll. Cardiol.* **39**: 726–731.
48. Shohet, R. V., *et al.* (2000). Echocardiographic destruction of albumin microbubbles directs gene delivery to the myocardium. *Circulation* **101**: 2554–2556.
49. Niwa, H., Yamamura, K., and Miyazaki, J. (1991). Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* **108**: 193–199.