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Improved gene transfer by direct plasmid injection associated with regeneration in mouse skeletal muscle

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Gene transfer into skeletal muscle via simple plasmid injection in vivo has many potential uses but these are severely constrained by the low efficiency of this technique. Muscle regeneration, induced by the myotoxic local anaesthetic bupivacaine, significantly increased gene expression following plasmid injection 3–7 days after bupivacaine treatment. Much of this effect can be attributed to uptake and expression of the plasmid by a greater number of muscle fibres, up to 9% of the mouse tibialis anterior muscle. A similar significant increase in expression was observed in the naturally regenerating muscle of the dystrophic *mdx* mouse when compared to the control C57Bl/10 strain.

Gene therapy; DNA injection; Plasmid DNA; Skeletal muscle; regeneration; bupivacaine; *mdx*

1. INTRODUCTION

Striated muscle has the almost unique ability to take up and express naked plasmid DNA. This property of skeletal muscle was first demonstrated by Wolff et al. in 1990, when they reported the expression of marker genes (chloramphenicol acetyltransferase [CAT] and β-galactosidase [β-gal]) following the direct injection of plasmid DNA solutions into rodent muscles [1]. Since then a number of investigators have documented this phenomenon in both skeletal [2,3] and cardiac [4–7] muscle of rodents, skeletal [8] and cardiac muscle [9,10] of other mammals, and the skeletal muscle of fish [11]. Other tissues do not appear to internalise and express significant amounts of naked plasmid DNA in vivo, with the possible exception of neural tissues [12].

The direct transfer technique has been proposed as a simple and cheap means of effecting somatic gene therapy, with little danger of genetic damage as the DNA appears to persist as an extrachromosomal circular plasmid [1,13]. Additionally, no immune reactions or adverse side effects were noted in a recent study in nonhuman primates, even after repeated treatments [8]. A further advantage is the injected plasmids continue to show expression for at least 19 months in mice [13].

Recently, direct DNA injection has been used successfully as a means of vaccinating mice against influenza [14]. Other uses of this gene transfer technique include: examination of skeletal and cardiac specific control elements of striated muscle genes [15], examination of the distribution of recombinant gene products in vivo [2], and testing constructs intended for expression in the muscles of transgenic mice (Wells et al., unpublished observations).

All of the above uses are hampered by the substantial variation in expression following a given injection (S.E.M. of 25–60% of the mean [3]) and the transfection of only a limited number of myofibres [2]. A number of variables affecting the technique have been identified, including different plasmid preparations, the solute used, innervation and muscle damage [3]. We recently reported two additional variables, namely the age and sex of the treated animal [16]. Variation in expression between injections appears to be reduced if the muscle is pre-treated with a sucrose solution [17]. In this report increased transfection and expression are associated with muscle regeneration, both natural and induced.

2. MATERIALS AND METHODS

2.1. *Plasmid production*

The experiments utilised the SV40 early promoter coupled to the chloramphenicol acetyltransferase reporter gene (pCAT-Control, Promega) and the RSV promoter coupled to the β-galactosidase reporter gene [18]. Plasmid DNA was prepared by standard caesium chloride centrifugation [19] and was checked for the purity of the supercoiled DNA by gel electrophoresis.

2.2. *Treatment of muscle with bupivacaine and injection of plasmid DNA*

C57Bl/10 ScSn mice, and the derived dystrophic *mdx* line [20], were reared in a barrier maintained colony with food and water available ad libitum. The myotoxic agent bupivacaine (0.5% Marcain, Astra Pharmaceuticals) was injected into the left tibialis anterior muscle at 2.5 μl/g body weight of mice anaesthetized with a mixture of Hypnorm and Hypnovel [21]. Three to twenty-one days later 50–100 μg of supercoiled plasmid DNA in PBS, final volume 50 μl, was injected through a 28-gauge needle (insulin syringe) into the middle of both left and right tibialis anterior muscles of the mice, again under general anaesthesia.

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2.3. *Chloramphenicol acetyl transferase assays*

Five days after plasmid injection the mice were sacrificed by cervical dislocation and the tibialis anterior muscle excised. The muscle was frozen in liquid nitrogen and ground to a fine powder using a pre-cooled mortar and pestle. The sample was then transferred to a 1.5 ml tube and resuspended in 200 μl of 0.25 M Tris-HCl, pH 7.8. The sample was subjected to three cycles of freezing and thawing (37°C), centrifuged using a Hereus microfuge at 13,000 rpm for 5 min, and the supernatant incubated at 65°C to inactivate endogenous acetyltransferases. After centrifugation at 13,000 rpm for 2 min, the supernatant was mixed with 100 μl of 1 M Tris-HCl, pH 7.8, 20 μl of acetyl coenzyme A (4 mg/ml) and 2 μl ^{14}C-labelled chloramphenicol (57.9 mCi/mmol, 0.05 mCi/ml). The mixture was incubated at 37°C for 2 h and the product was resolved by thin-layer chromatography [19]. X-Ray film was exposed overnight to the TLC plates. CAT activity was measured as the percentage of chloramphenicol conversion to acetylated forms using a laser densitometer (Chromoscan 3, Joyce-Loebl).

2.4. *β-galactosidase histochemistry*

Nine days after plasmid injection the mice were sacrificed by cervical dislocation. After dissection the tibialis anterior muscles were fixed for 30 min in 0.5% glutaraldehyde in phosphate buffer (100 mM, pH 7.0, 1 mM $MgCl_2$, 0.02% Nonidet-P40). After fixation muscles were sliced transversely in approximately 1–2 mm sections, fixed for a further 15 min, and then rinsed in 3 changes of phosphate buffer. Muscle slices were stained to reveal β-galactosidase activity with X-gal solution (10 mM PO_4 buffer, 1 mM $MgCl_2$, 0.15 M NaCl, 33 mM $K_3Fe(CN)_6$, 33 mM $K_4Fe(CN)_6$, 0.1% X-galactosidase in dimethylformamide). After incubation at 37°C for 45 min the number of β-gal positive fibres could be counted using a dissecting microscope. Counts were confirmed by dehydrating, wax embedding, and sectioning the muscle slices.

3. RESULTS

3.1. *Bupivacaine pre-treatment enhances expression*

Fourteen-week-old male C57Bl/10 mice were injected with bupivacaine in the left tibialis anterior muscle. Three, seven or twenty-one days later 100 μg of SV40-CAT plasmid in 50 μl of PBS was injected into the left and the right tibialis anterior muscles. Five days later the mice were sacrificed and the muscle extract was assayed for CAT activity (Fig. 1). Mice injected three days after bupivacaine treatment showed significantly increased CAT activity in the pre-treated muscle compared to the contralateral control ($n = 7$, $P < 0.02$). Mice treated with bupivacaine seven days prior to the injection of plasmid showed similar dramatic increases in expression on the treated side compared to the contralateral control ($n = 5$, $P < 0.001$) but this effect was lost in muscles treated twenty-one days before plasmid injection ($n = 5$, N.S.). Comparisons analyzed with correlated t-tests [22].

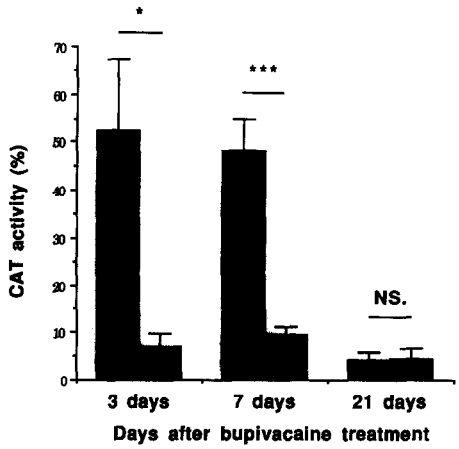

Fig. 1. Mice, with the left tibialis anterior muscle treated with bupivacaine 3, 7 or 21 days earlier, were injected with 100 μg of SV40-CAT plasmid. Significant differences in CAT activity were seen between the pre-treated (black bars) and the untreated (striped bars) muscles at 3 and 7 days (*$P < 0.02$, ***$P < 0.001$) but not at 21 days.

3.2. *Bupivacaine pre-treatment enhances transfection*

Eleven-week-old male C57Bl/10 mice were injected with bupivacaine in the left tibialis anterior muscle. Five days later 100 μg of RSV-βgal plasmid in 50 μl PBS was injected into the left and the right tibialis anterior muscles. Nine days later the mice were sacrificed and the muscles stained for fibres expressing β-galactosidase (Fig. 2). Muscles pre-treated with bupivacaine had 3 times the number of β-galactosidase positive fibres as the contralateral controls ($n = 4$, $P < 0.05$, correlated t-test). In one animal 9% of the muscle fibres were transfected by this single treatment.

3.3. *Natural regeneration enhances expression*

Eight-week-old male C57Bl/10 and *mdx* mice were injected with bupivacaine in the left tibialis anterior muscle. Five days later 50 μg of SV40-CAT plasmid in 50 μl PBS was injected into the left and the right tibialis anterior muscles. Five days later the mice were sacrificed and the muscle extract was assayed for CAT activity (Fig. 3). As before, pre-treatment with bupivacaine enhanced gene expression in the C57Bl/10 mice. This bupivacaine effect was not seen in the *mdx* strain but these mice show significantly higher gene expression without pre-treatment compared to the C57Bl/10 mice ($n = 9$, $P < 0.05$, t-test).

4. DISCUSSION

Bupivacaine, a local anaesthetic, is myotoxic when injected into skeletal muscle [23]. It causes increased membrane permeability to calcium ions which leads to fibre hypercontraction and necrosis [24]. Innervation, blood supply, satellite cells and connective tissues are not significantly disturbed by this treatment [25]. The necrotic muscle regenerates very rapidly; myotubes are apparent by three days after treatment, much of the fibre architecture is re-established by seven days and the muscle phenotype is fully restored by 21 days [26,27]. Hence, it is not surprising that enhanced plasmid expression, observed during regeneration, was not seen when plasmids were injected into muscles 21 days after bupivacaine treatment.

The improved efficiency of plasmid gene transfer into

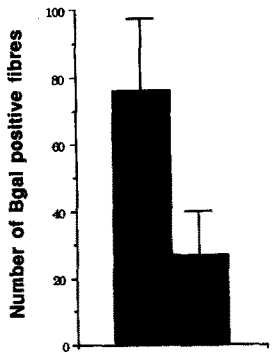

Fig. 2. Mice, with the left tibialis anterior muscle treated with bupivacaine 5 days earlier, were injected with 100 μg of RSV-βgal plasmid. A significant difference in the number of β-gal positive fibres was observed when comparing the pre-treated (black bar) and the untreated (striped bar) muscles ($P < 0.05$).

regenerating muscle may be due to one or more of a number possible factors. Muscle destruction, inflammation and the formation of small myotubes may allow for an improved distribution of the injected plasmid and increased access of the DNA to the sarcolemma of the muscle. Variation in the efficiency of gene transfer between individual injections has been attributed to differences in plasmid distribution within the muscle [17]. Alternatively, the process of regeneration may be associated with a higher density of the putative receptors involved in the uptake of the DNA. DNA uptake by skeletal muscle has been postulated to be an active uptake process involving caveolae [28]. Translocation to the nucleus may also be affected by regeneration, perhaps associated with altered lysosomal dynamics. Finally, muscle fibre growth may lead to increased transcription of the plasmid DNA, as suggested by Wells and Goldspink [16].

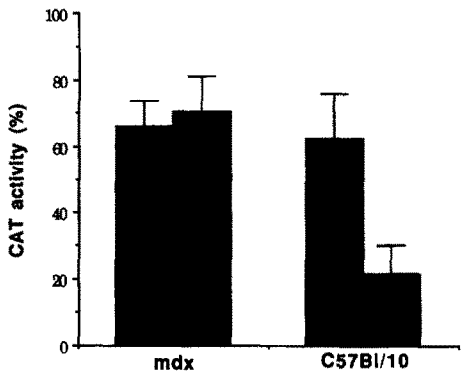

Fig. 3. C57Bl/10 and *mdx* mice, with the left tibialis anterior muscle treated with bupivacaine 5 days earlier, were injected with 50 μg of SV40-CAT plasmid. A significant difference in CAT activity was seen between the untreated muscles of the C57Bl/10 and *mdx* mice (striped bars, $P < 0.05$). CAT expression increased with bupivacaine pre-treatment in the C57Bl/10 mice but bupivacaine did not increase expression in the *mdx* mice (black bars).

Regardless of the mode of action, regeneration is none the less a very useful means of increasing the efficiency of gene transfer with this plasmid injection method. Possible applications for natural or induced regeneration as an adjunct to gene transfer include: gene therapy, DNA based vaccination protocols, and for examination of muscle gene function in vivo. The latter will become more important as differences are increasingly recognised between gene activity in vitro, generally in transformed permanent cell lines, and performance in vivo [29,30]. As such, this simple technique may in part substitute for the expensive and time-consuming production of transgenic mice for the in vivo analysis of gene function.

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REFERENCES

[1] Wolff, J.A., Malone, R.W., Williams, P., Wang, C., Acsadi, G., Jani, A. and Felgener, P.L. (1990) Science 247, 1465–1468.
[2] Acsadi, G., Dickson, G., Love, D.R., Jani,A., Walsh, F.S., Gurusinghe, A., Wolff, J.A. and Davies, K.E. (1991) Nature 352, 815–818.
[3] Wolff, J.A., Williams, P., Acsadi, G., Jiao, S.S., Jani, A. and Wang, C. (1991) Biotechniques 11, 474–485.
[4] Acsadi, G., Jiao, S.S., Jani, A., Duke, D., Williams, P., Wang, C. and Wolff, J.A. (1991) New Biol. 3, 71–81.
[5] Buttrick, P.M., Kass, A., Kitsis, R.N., Kaplan, M.L. and Leiwand, L.A. (1992) Circ. Res. 70, 193–198.
[6] Kitsis, R.N., Buttrick, P.M., McNally, E.M., Kaplan, M.L., and Leinwand, L.A. (1991) Proc. Natl. Acad. Sci. USA 88, 4138–4142.
[7] Lin, H., Parmacek, M.S., Morle, G., Bolling, S. and Leiden, J.M. (1990) Circulation 82, 2217–2221.
[8] Jiao, S.S., Williams, P., Berg, R.K., Hodgeman, B.A., Liu, L., Repetto, G. and Wolff, J.A. (1992) Human Gene Therapy 3, 21–33.
[9] Gal, D., Weir, L., Leclerc, G., Pickering, J.G., Hogan, J. and Isner, J.M. (1993) Lab. Invest. 68, 18–25.
[10] von-Harsdorf, R., Schott, R.J., Shen, Y.T., Vatner, S.F., Mahdavi, V. and Nadal-Ginard, B. (1993) Circ. Res. 72, 688–695.
[11] Hansen, E., Fernandes, K., Goldspink, G., Butterworth, P., Umeda, P.K. and Chang, K.-C. (1991) FEBS Lett. 290, 73–76.
[12] Sahenk, Z., Seharaseyon, J., Mendell, J.R. and Burghes, A.H. (1993) Brain Res. 606, 126–129.
[13] Wolff, J.A., Ludtke, J.J., Acsadi, G., Williams, P. and Jani, A. (1992a) Hum. Mol. Genet. 1, 363–369.
[14] Ulmer, J.B., Donnelly, J.J., Parker, S.E., Rhodes, G.R., Felgner, P.L., Dwarki, V.J., Gromkowski, S.H., Deck, R.R., DeWitt, C.M., Friedman, A., Hawe, L.A., Leander, K.R., Martinez, D., Perry, H.C., Shiver, J.W., Montgomery, D.L. and Liu, M.A. (1993) Science 259, 1745–1749.
[15] Vincent, C.K., Gualberto, A., Patel, C.V. and Walsh, K. (1993) Mol. Cell. Biol. 13, 1264–1272.
[16] Wells, D.J. and Goldspink, G. (1992) FEBS Lett. 306, 203–205.
[17] Davis, H.L., Whalen, R.G. and Demeniex, B.A. (1993) Human Gene Therapy 4, 151–159.
[18] Norton, P.A. and Coffin, J.M. (1985) Mol. Cell. Biol. 5, 281.
[19] Sambrook, J., Fritsch, T. and Maniatis, T. (1989) Molecular Cloning, a Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, NY.
[20] Bulfield, G., Siller, W.G., Wight, P.A.L. and Moore, K.J. (1984) Proc. Natl. Acad. Sci. USA 81, 1189–1192.

[21] Flecknell, P.A. (1987) Laboratory Animal Anaesthesia, Academic Press, London.
[22] Bailey, N.T.J. (1981) Statistical Methods in Biology, 2nd edn., Hodder and Stoughton, London.
[23] Benoit, P.W. and Belt, W.D. (1970) J. Anat. 107, 547–556.
[24] Nonaka,I., Takagi, A., Ishiura, S., Nakase, H. and Sugita, H. (1983) Acta Neuropathol. 60, 167–174.
[25] Alameddine, H.S., Hantai, D., Dehaupas, M. and Fardeau, M. (1991) Neuromusc. Disorders 1, 143–152.
[26] Grounds, M.D. and McGeachie, J.K. (1992) Muscle Nerve 15, 580–586.
[27] Dunckley, M., Wells, D.J., Walsh, F.S. aand Dickson, G. (1993) Hum. Mol. Genet. 2, 717–723.
[28] Wolff, J.A., Dowty, M.E., Jiao, S., Repetto, G., Berg, R.K., Ludtke, J.J. and Williams, P. (1992) J. Cell Sci. 103, 1249–1259.
[29] Johnson, J.E., Wold, B.J. and Hauschka, S.D. (1989) Mol. Cell. Biol. 9, 3393–3399.
[30] Jackson, R.J. (1993) Cell 74, 9–14.