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Age and sex influence expression of plasmid DNA directly injected into mouse skeletal muscle

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Direct injection of plasmid DNA into the skeletal muscle has been proposed as a means of effecting somatic gene therapy. We examined the influence of age and sex on the level of expression of an SV40-CAT construct injected into mouse muscle. Age markedly affected expression, with peak values in the 4–6 week age class which were significantly higher than in animals older than 10 weeks. Sex also altered expression, with higher levels of CAT activity seen in males compared to females. We conclude that the rate of growth is important in determining levels of expression of directly injected DNA.

Gene therapy; DNA injection; Plasmid DNA; Skeletal muscle; Sex; Age

1. INTRODUCTION

Since the original report by Wolff et al. [1] demonstrating the expression of reporter genes following the direct injection of plasmid DNA into rodent muscle, a number of papers have documented the same phenomenon in both skeletal [2,3] and cardiac [4–6] muscle of rodents, skeletal muscle of other mammals [7] and the skeletal muscle of fish [8].

The direct transfer technique has been proposed as a means of effecting somatic gene therapy with little danger of genetic side effects as the DNA appears to persist as an extrachromosomal circular plasmid [1]. Additionally, no immune reactions or adverse side effects were noted in a recent study in non-human primates, even after repeated treatments [7]. A further advantage is that the injected plasmids continue to show expression for at least one year [9]. Unfortunately, application is hampered by variable results and the transfection of only a limited number of myofibers [2]. The current report examines two possible causes of variation, namely the age and sex of the treated animal. This complements a recent paper examining some of the other factors influencing the expression of injected plasmids [3].

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2. MATERIALS AND METHODS

2.1. *Plasmid production*

The experiments utilised the SV40 early promotor coupled to the chloramphenicol acetyltransferase reporter gene (pCAT–Control, Promega). Plasmid DNA was prepared using Quiagen columns (Diagen) following the supplier's protocol and was checked for concentration and quality by UV spectrophotometry and agarose gel electrophoresis [10].

2.2. *Injection of plasmid DNA in vivo*

C57B1/10 ScSn mice were reared under a conventional management system with food and water available ad libitum. 100 μg of plasmid DNA in 20% sucrose, final volume 100 μl, was injected through a 27 gauge needle into the middle of the quadriceps muscle of mice anaesthetised with Avertin or a mixture of Hypnorm and Hypnovel [11]. No skin incision was made and care was taken to identify and avoid the fat pad overlying the antero-proximal portion of the quadriceps muscle, particularly in older mice. Animals of a range of ages were injected with each plasmid preparation.

2.3. *Chloramphenicol acetyltransferase assays*

72 h later the mice were sacrificed by cervical dislocation and the entire quadriceps 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 Heraeus 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 4 h and the product resolved by thin layer chromatography [10]. 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).

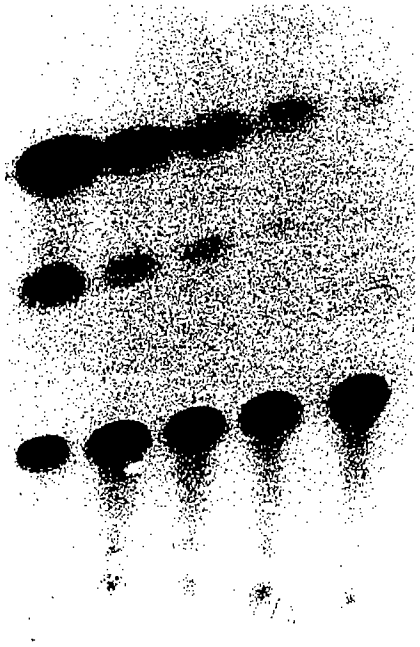

Fig. 1. A typical CAT assay with samples taken 72 h after injection of mouse quadriceps muscle with 100 μg of pSVCAT. Lane A, 8-week-old male, 79% CAT activity; lane B, 11-week-old male, 25.5% CAT activity; lane C, 13-week-old male, 16.3% CAT activity; lane D, 15-week-old male, 4% CAT activity; lane E, 20-week-old male, 0.3% CAT activity.

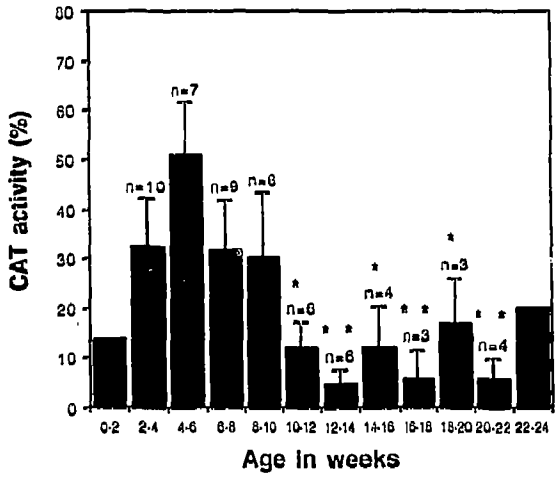

Fig. 2. CAT activity measured as the percentage of chloramphenicol conversion to acetylated forms, of samples taken 72 h after injection of 100 μg of pSVCAT into the quadriceps of male and female mice of various ages. T-Bars represent the standard error. One way ANOVA, $P<0.05$, for age. Expression is significantly lower in the 10–22-week-old groups compared to the 4–6-week-old group (paired t-tests, $^{*}P<0.05$, $^{**}P<0.01$).

3. RESULTS

3.1. *Influence of age*

The animals showed no signs of locomotor impairment or other adverse reactions following treatment. Considerable individual variation in expression of the injected DNA was observed within each age group. Despite this variation, animal age had a marked effect on the degree of plasmid expression (Fig. 1). Younger animals demonstrated higher levels of CAT activity than older animals (Fig. 2, ANOVA $P<0.05$). The highest levels of expression were obtained in the 4–6 week age group, significantly higher than in animals older than 10 weeks. Very young animals (<2 weeks old) do not show good expression and appear to thrive poorly following treatment, either due to muscle damage or the effects of anaesthesia. Therefore only one very young animal was included in this study.

3.2. *Influence of sex*

The sex of the treated animal appears to influence the degree of expression of the injected plasmid DNA, with males exhibiting greater expression than females, although the difference is only statistically significant at 2–4 and 10–12 weeks. The effect of age is clearly observed in males but age does not appear to have such a significant effect in females, due to low levels of expression and considerable variation (Fig. 3A, males ANOVA $P<0.05$; Fig. 3B, females ANOVA not significant). However, the trend appears to be the same between the two sexes.

4. DISCUSSION

The major finding of these studies is the observation that age significantly influences the level of expression of the injected plasmid. Higher yields of the reporter

Table I

Study	Species (strain)	Age	Sex
Wolff et al. [1]	Mouse (Balb/c)	5–6 weeks	Mixed
Lin et al. [5]	Rat (SD)	6–11 weeks (250 g)	?
Acsadi et al. [2]	Mouse (mdx)	4–6 weeks	?
Acsadi et al. [4]	Mouse (Balb/c)	?	?
	Mouse (Balb/c)	4 weeks	?
	Rat (SD)	Adult	?
	Rat (nude SD)	?	?
	Rat (SD)	150–280 g	?
Hansen et al. [8]	Carp	Young (10 cm)	?
	Carp	Old (20 cm)	?
Kitsis et al. [6]	Rat (Wistar)	Adult	Female
Wolff et al. [3]	Mouse (Balb/c)	6 weeks	Mixed
	Rat	5–7 weeks	?
	Rat	1 day	?
Jiao et al. [7]	Rhesus monkey	5–11 years	?
	Cat	?	?
	Rat	?	?

SD = Sprague–Dawley

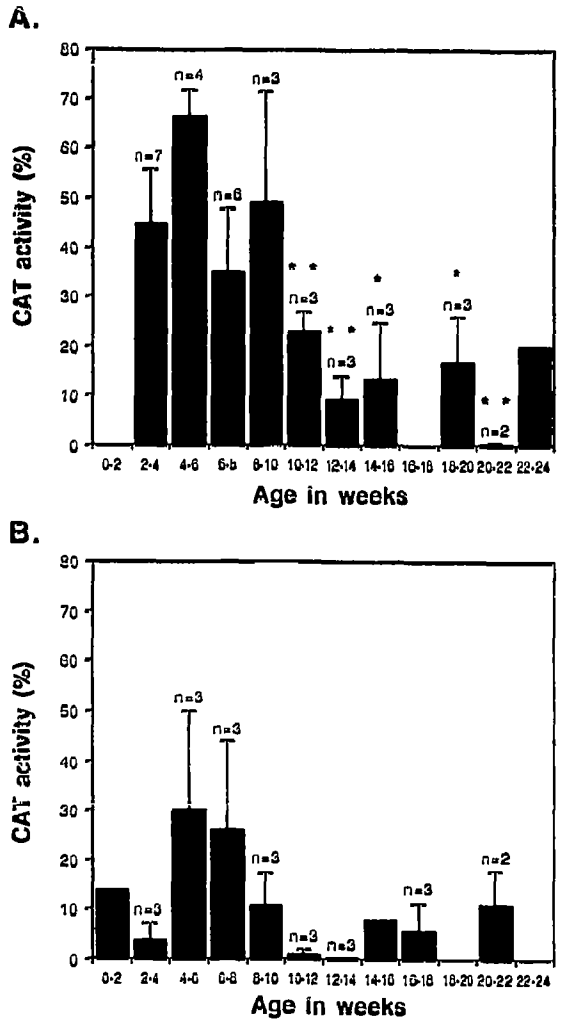

Fig. 3. (A) CAT activity measured as the percentage of chloramphenicol conversion to acetylated forms, of samples from treated male mice of various ages. T-Bars represent the standard error. One way ANOVA, $P<0.05$, for age. Expression is significantly lower in the 10–16 and 18–22-week-old groups compared to the 4–6-week-old group (paired t-tests, $^{*}P<0.05$, $^{**}P<0.01$). (B) CAT activity of samples from treated female mice of various ages. T-Bars represent the standard error. One way ANOVA, not significant.

protein were obtained when young animals (ranging from 2–10 weeks old) were treated, particularly those 4–6 weeks old. Perhaps fortuitously, this is also the age range used in most of the previously reported mouse studies (e.g. [1–4]). Regrettably, a number of studies have failed to record the age and sex of the treated animals (see Table I). Certainly, future studies should carefully consider these variables. For example, the comparative study conducted by Jiao and colleagues [7] used adult primates in comparison with young mice (from [3]) and the conclusions should be regarded with caution.

An explanation of differences due to age and sex may lie in the growth rate of the treated animals. Mice show rapid growth from three to approximately 10 weeks of age followed by a marked reduction in rate for the following weeks of life (e.g. [12]). This might affect plasmid expression either by increased uptake of the plasmid or by increased transcription/translation per gene copy. Females grow less rapidly than males and this could explain the decreased expression of injected DNA in females compared to males. Variation in individual growth rates and response to the stress of treatment might well account for some of the marked individual-to-individual variation recorded in these and other experiments. Age effects are not confined to mice; increased CAT expression has been recorded in young compared to older fish [8].

In conclusion, this report extends the number of variables known to affect the direct injection technique. Future studies should pay more attention to the age, sex and physiological state of experimental subjects. The strain and husbandry conditions should also be recorded as these variables will affect growth rate. Most significantly, the results suggest that direct muscle gene therapy would be most successful in younger patients.

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