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Regeneration after free muscle grafting in normal and dystrophic (*mdx*) mice

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Summary

Soleus muscles from C57BL/10 and *mdx* mice were isotransplanted to induce a cycle of degeneration/regeneration. Sixty days post-surgery, transplanted and contralateral soleus muscles were removed for mechanical and biochemical analyses. The regeneration which occurs after transplantation, induces in both *mdx* and C57BL/10 soleus muscles a decrease in maximal isometric force, together with an increase of the velocity of contraction. This increase in velocity is accompanied by the expression of typically fast-type myosin heavy chains. Thus degeneration/regeneration of both *mdx* and normal mice are very similar, causing a shift towards physiologically 'faster' muscle. Previous physiological and biochemical studies of *mdx* muscles have shown that *mdx* muscle is shifted towards 'slower' muscle compared to normal mice. One explanation of these findings was that the degeneration/regeneration cycles inherent in dystrophin-deficient *mdx* muscle causes a shift towards 'slow'. Our results argue against this hypothesis: degeneration/regeneration in both normal and *mdx* mice causes a shift towards 'fast'.

Introduction

Both human Duchenne muscular dystrophy (DMD) and an animal model, the *mdx* mouse (Bulfield et al. 1984), show repeated cycles of muscle degeneration/regeneration caused by dystrophin deficiency (Coulton et al. 1988; Marshall et al. 1989).

Repeated cycles of degeneration/regeneration could result in an incomplete muscle regeneration due to dystrophin deficiency. The replacement of contractile muscle fibres by fat or connective tissue would account for the muscle weakness characteristic of the disease.

In the mouse, a cycle of degeneration and regeneration can be experimentally induced in a whole muscle by performing free muscle grafting in order to investigate whether or not dystrophin deficiency depresses the regeneration process of the muscle.

Another well known feature of DMD is an increased proportion of type 1 fibres (Dubowitz and Brooke 1973) bringing on a slower velocity of muscle contraction (McComas et al. 1971; Edwards et al. 1987).

Aging of *mdx* soleus muscle increases the proportion of slow-twitch type 1 fibres (Carnwath and Shotton 1987; Anderson et al. 1988; Marshall et al. 1989) and decreases its velocity of contraction (Anderson et al. 1988; Maréchal and Beckers-Bleukx 1991). This effect of aging might be a consequence of the repeated cycles of degeneration/regeneration.

In this work, we investigate whether regeneration is depressed by dystrophin deficiency and whether changes in slow-twitch fibres proportion and muscle velocity of contraction can be attributed to repeated degeneration/regeneration cycles or to some other consequence of dystrophin deficiency. To do this, we have induced a cycle of degeneration/regeneration in normal (C57BL/10 ScSn) and dystrophic mice (*mdx*) by orthotopic isotransplantation and, after regeneration, have analyzed the mechanical and biochemical properties of the muscles. Transplanted normal and *mdx* muscles show similar changes after regeneration, suggesting that dystrophin deficiency does not depress the regeneration process. However, transplanted normal muscles show the opposite changes to those which occur in the non-transplanted *mdx* soleus: an increase in velocity of contraction together with a decrease in

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the proportion of myosin heavy chain 1 (MHC1), a protein typical of type 1 fibres. We also observed that isotransplanted *mdx* muscles undergo a similar increase in velocity of contraction. We conclude, therefore, that *mdx* muscles are physiologically and biochemically slower than normal for reasons other than their spontaneous cycles of degeneration/regeneration.

Materials and methods

Mouse strains

Myopathic mice (*mdx*, $n = 12$) and mice of their control strain (C57BL/10, $n = 16$) were housed at Charing Cross and Westminster Medical School, London.

Grafting

Donor mice of each strain were killed by cervical dislocation and the soleus muscles were dissected with a maximum length of tendons attached. Prior to removal, a knot of surgical silk was tied around the proximal and distal tendons and the inter-knot distance with the foot at right angles to the lower limb was measured and recorded for each individual muscle. This was taken as the standard length of the muscle. The muscles were kept 1–3 h in Krebs solution (mM: NaCl 118, $NaHCO_3$ 25, KCl 5, $MgSO_4$ 1, KH_2PO_4 1, glucose 5, $CaCl_2$ 2.5; pH 7.3; gassed with 95% O_2 and 5% CO_2; 20°C) prior to grafting into host mice. Just before grafting, the muscles were coloured with a blue tatoo dye in order to facilitate the distinction of the grafts from adjacent host muscles after regeneration.

The host mice (circa 60 days old) were anesthetized by subcutaneous injection with 0.1 ml/10 g body weight of a mixture of Fentanyl (5 mg/ml) and Hypnomidate (2 mg/ml) in a ratio of 1/5.

One of their soleus muscles was excised leaving a maximum length of tendons in situ. The muscle of a donor mouse was placed orthotopically in a host mouse of the same strain and tied to host tendons at the same muscle length as that measured in the donor mouse.

Adjacent muscles and skin were sutured with surgical silk (SSC 8-0 and 7-0, respectively).

Sixty days post-surgery, the mice were killed and both grafts and contralateral muscles (which served as control) were dissected for mechanical and biochemical analyses.

The graft is considered to be stabilized after 60 days as in minced muscles of mice, the fibres look quite mature 30 days post-injury (Mufti 1977) and most of histological and contractile properties of soleus muscle graft in the rat are stabilized 30–40 days after grafting (Carlson et al. 1979).

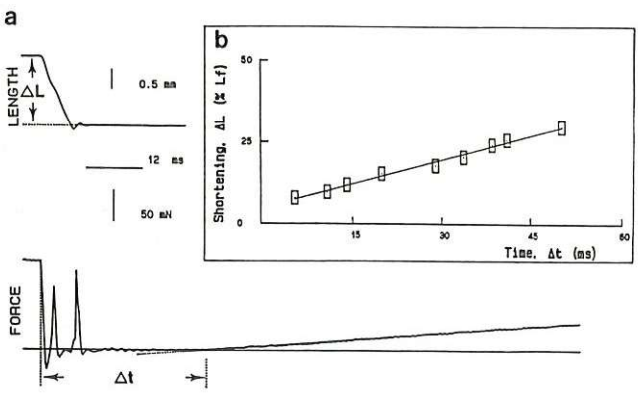

Fig. 1. Determination of the unloaded maximum velocity of shortening. (a) Variation of length (ΔL) and force in function of time. Δt is the time necessary for the muscle to take up a force. (b) Plot of the distance of shortening (ΔL) in function of the time (Δt).

Mechanical analyses

The muscles were mounted on an electromagnetic puller described by Maréchal and Plaghki (1979) and kept at 20 ± 1°C in a Krebs solution oxygenated by 95% O_2 and 5% CO_2. The length of the muscles was adjusted so that the resting force was $5\% \pm 2$ of the maximum isometric force. The muscle fibre length (Lf) was evaluated by microscopic measurements of a few superficial fibres and divided by the muscle length (Lo). The ratio Lf/Lo was 0.68, a value which is in fair agreement with Brooks and Faulkner (1988) data on mouse soleus muscle. We analyzed the force of the twitch (Ft), the force of the twitch by unit muscle area (Pt = Ft * Lf/muscle weight (Wm) (density = 1)), time to peak (TP) and half relaxation time (HRT), the maximal isometric force (Fo) of a fused tetanus (50 stim/s during 1 s), the stress (So), which is Fo normalized per unit muscle area (So = Fo*Lf/Wm (density = 1)). The unloaded maximum velocity of shortening (Vu) was measured by the slack-test method (Edman 1979) (Fig. 1): the muscle stimulated in isometric tetanus was released at a constant velocity (286 mm/s), when the force had reached a plateau, for 9 different distances ranging from 0.2 to 2 mm. The muscle was permitted a rest of 3 min between each tetanus. During the release, the force falls to zero. The time necessary for the muscle to take up a force was plotted against the distance of shortening. The slope of the line fitted through the experimental points gave the value of Vu (Lf/s).

Mechanical data were collected on a PC-AT computer by an A/D conversion card (RTI-815) and analyzed with an interactive program written in Truebasic.

After mechanical analyses, the muscles were weighed and frozen at standard muscle length in isopentane cooled with liquid nitrogen.

Biochemical analyses

Each frozen muscle (approximately 12 mg) was ground in a cooled mortar and extracted on ice in 50 μl (transplant muscles) or 100 μl (control muscles) of Guba–Straub solution (mM: NaCl 300, NaH_2PO_4 100, $MgCl_2$ 1, $Na_4P_2O_7$ 10, Na_2HPO_4 50, Na azide 0.1%; pH 6.5) with 0.1% (vol/vol) of β-mercaptoethanol. After centrifugation for 10 min at 20000 rpm, the supernatant was mixed with 1 vol. glycerol.

SDS polyacrylamide–gel electrophoresis (SDS–PAGE) was performed as described by Danieli-Betto et al. (1986), with the inclusion of 33% (weight/vol.) glycerol in both separating and stacking gels (Biral et al. 1988). The sample with glycerol was diluted in a buffer (mM: Tris 62.5, SDS 2.3%, glycerol 10%, β-mercaptoethanol 5%; pH 6.8) at 1/400 for the control and 1/200 for the transplant muscles. The electrophoretic run (10 mA) was performed with 5 and 10 μl of the diluted extracts (20–80 ng/μl of proteins), using β-galactosidase as a reference for myosin heavy-chain electrophoretic mobility (R_f).

Staining was performed overnight in a Coomassie blue solution (Serva G250 40 mg; perchloric acid 70% 3 ml in 100 ml H_2O) and gels were destained in 7% acetic acid.

Gels were analyzed by densitometry. Densitograms display a peak for each myosin heavy chain. The R_f values of the peaks were expressed relatively to the R_f of β-galactosidase (set at 100). The proportion of each isoform, as evaluated by the area of each peak on the densitograms, was expressed as a fraction of the total area of skeletal myosin heavy chains.

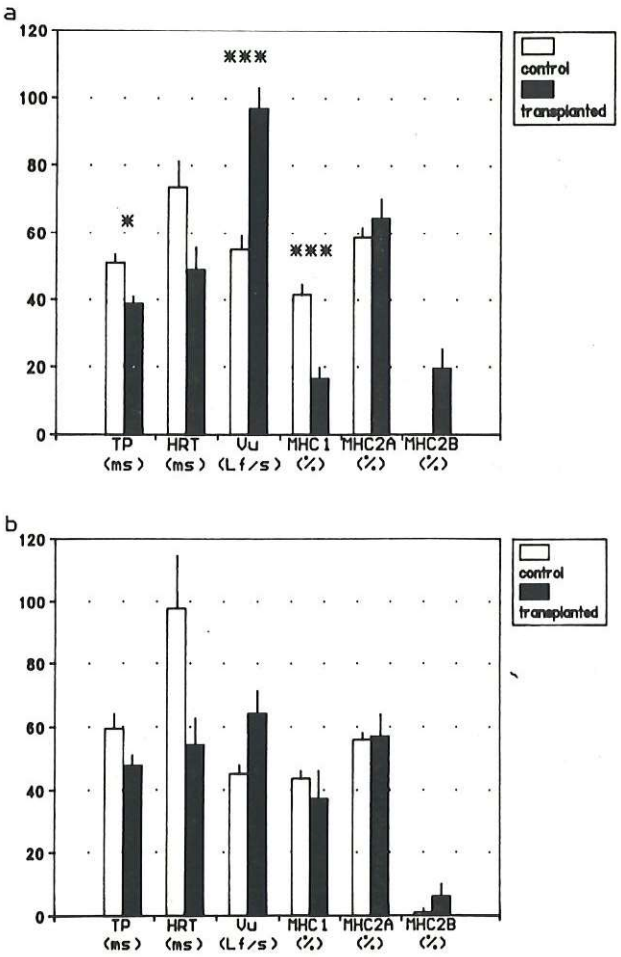

Fig. 2. Mechanical and biochemical parameters of control and transplanted soleus muscle of (a) C57BL/10 mice and (b) *mdx* mice. The unloaded maximum velocity of shortening (Vu) was multiplied 10 times to fit the graph. Vertical bars are the SEM. * $P < 0.05$; *** $P < 0.001$.

Results

Normal soleus muscles of C57BL / 10 and mdx mice

Figures 2a and b show our results from normal soleus muscles of 7 C57BL/10 and 6 *mdx* mice: they are in agreement with those reported by Maréchal and Beckers-Bleukx (1991). Compared to C57BL/10 soleus muscles, twitch time to peak and half relaxation time are higher and unloaded velocity of shortening is lower in *mdx* soleus muscles. In parallel, the *mdx* soleus muscles have a slightly higher proportion of slow type MHC (MHC1), in agreement with the finding that these muscles are slower in contracting than the C57BL/10 soleus muscles.

Transplantation of C57BL / 10 soleus muscles

Table 1 shows the morphometric and maximal isometric force parameters in control ($n = 7$) and transplanted ($n = 9$) soleus muscles of C57BL/10 mice. Transplantation induces a significant decrease in muscle length (Lo) and a decrease in maximal isometric force (Fo). The maximal isometric stress (So) in transplanted soleus is 42% lower than in control muscles. The force of the twitch is not different from control values. However, the kinetic parameters of the twitch were altered (Fig. 2a): time to peak and half relaxation time are shorter in transplanted muscles than in con-

TABLE 1

ISOMETRIC FORCE OF CONTROL AND TRANSPLANTED SOLEUS MUSCLES OF C57 MICE

C57 mice	Control (mean ± SEM) ($n = 7$)		Transplanted (mean ± SEM) ($n = 9$)
Lo (mm)	9.8 ± 0.2	**	7.2 ± 0.6
Wm (mg)	12.6 ± 0.7		11.0 ± 0.8
Fo (mN)	222 ± 10		167 ± 24
So (mN/mm^2)	120 ± 8	***	70 ± 8
Ft (mN)	26 ± 3		31 ± 7
Pt (mN/mm^2)	14 ± 2		13 ± 2

** $P < 0.01$; *** $P < 0.001$.

TABLE 2

ISOMETRIC FORCE OF CONTROL AND TRANSPLANTED SOLEUS MUSCLES OF *mdx* MICE

mdx mice	Control (mean ± SEM) ($n = 6$)		Transplanted (mean ± SEM) ($n = 6$)
Lo (mm)	10.0 ± 0.3	**	7.4 ± 0.6
Wm (mg)	16.1 ± 0.7	*	11.4 ± 2.0
Fo (mN)	264 ± 21	*	164 ± 33
So (mN/mm^2)	115 ± 14	*	70 ± 10
Ft (mN)	32 ± 7		19 ± 6
Pt (mN/mm^2)	14 ± 3		7 ± 2

* $P < 0.05$; ** $P > 0.01$.

trol muscles. The maximal velocity of unloaded shortening is significantly higher in grafted soleus muscles. The proportions of myosin heavy chains (Fig. 2a) are modified towards higher percentages of fast myosin heavy chains: decrease of the proportion of slow MHC (MHC1) and the expression in transplanted muscles of a typically fast MHC (MHC2B) which is not seen in control muscles.

Transplantation of mdx soleus muscles

Transplantation in *mdx* mice (Table 2) induces effectively the same modifications as in C57BL/10 mice: a decrease in muscle length and weight, a decrease in maximal isometric force and a lower maximal isometric stress in transplanted soleus muscles than in control muscles.

Figure 2b shows that the kinetic parameters and proportions of myosin heavy chains in *mdx* ($n = 6$) were modified to a lesser extent but in the same direction as in C57BL/10 mice: shorter time to peak and half relaxation time, higher unloaded maximal velocity of contraction in transplanted soleus than in non-transplanted muscles together with a slightly lower proportion of MHC1 and the presence of MHC2B.

Discussion

The values of maximal isometric force and stress developed by the *mdx* soleus are similar to those developed by C57 soleus muscle, both in control and in transplanted muscles (Tables 1 and 2), confirming our previous results (Moens et al. 1990).

Following muscle transplantation in mouse, the fibres degenerate and subsequently regenerate, except for a few fibres located at the periphery (Roberts et al. 1989). The myogenic precursor cells (mpc) originate from the transplanted muscle (Neerunjun and Dubowitz 1974) but some mpc may come from host muscles (Morgan et al. 1987, 1988). The decrease in maximal isometric stress (So) seen in regenerated muscles could be due to the proliferation of connective tissue or fat, as reported in the case of transplanted rabbit (Burton et al. 1989), rat muscle (Segal et al. 1986) and minced rat muscle (Ghins et al. 1986).

All the kinetic parameters of transplanted C57 and *mdx* soleus muscles indicate that the velocity of the muscle is higher after 60 days of regeneration. We have previously shown (Moens et al. 1992) that this higher velocity of contraction is attributable to the presence in transplanted soleus of a variety of myosin containing MHC2A with fast light chains ($LC1_f$, $LC2_f$, $LC3_f$), which is absent from normal and *mdx* soleus muscles. In free muscle grafts, the innervation is cut and the regenerating muscle must be reinnervated. The muscle graft might be reinnervated either by its normal nerve or by branches from nerves supplying adjacent muscles. Since all of the neighbouring muscles are predominantly fast, the modifications in the isomyosin pattern of transplanted soleus muscles might be due to reinnervation from 'fast' motor nerves.

Similar changes in mechanical and biochemical properties of grafted muscles are observed in both *mdx* and C57 mice, showing that the regeneration process is the same in the two strains and is not modified by dystrophin deficiency.

We found that both regenerated and non-manipulated soleus muscles of *mdx* contract more slowly and have a higher proportion of slow type of myosin heavy chains compared to C57 muscles.

These results suggest, in agreement with one of the hypotheses proposed by Carnwath and Shotton (1987), that the shift to 'slow' observed in dystrophin-deficient *mdx* (and human) muscle occurs by favoring the differentiation of immature regenerating fibres into slow-type fibres rather than into fast-type fibres.

After grafting, mechanical (kinetic) and biochemical parameters are modified in the opposite direction from those induced by dystrophin-deficiency in *mdx* muscle. Thus the process of degeneration/regeneration cannot account for the slower velocity of contraction and the higher proportion of MHC1 or type 1 fibres seen in dystrophin-deficient *mdx* soleus muscles. The phenomenon must therefore arise via some other mechanism which is altered by lack of dystrophin.

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