



# An evaluation of leukaemia inhibitory factor as a potential therapeutic agent in the treatment of muscle disease

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## Abstract

The exogenous delivery of growth factors and cytokines is a potential therapeutic strategy to alleviate the degenerative effects of primary inherited myopathies such as Duchenne muscular dystrophy. The *mdx* mouse diaphragm is a model for examining the progressive degeneration of dystrophic muscle. We have delivered leukaemia inhibitory factor to the *mdx* diaphragm using slow release alginate gels. Previous studies have reported an improvement in the histology of *mdx* diaphragms after delivery of leukaemia inhibitory factor in a similar manner, but little attention has been paid to the mechanism by which leukaemia inhibitory factor acts. We have used autoradiography to examine cell proliferation, Evans Blue Dye to examine myofibre damage, and morphometric analysis to examine histology in leukaemia-inhibitory-factor-treated diaphragms and compared them with untreated *mdx* and normal C57B110/ScSn diaphragms. Autoradiography showed that although myoblast proliferation was significantly increased in leukaemia inhibitory factor-treated *mdx* diaphragms, leukaemia inhibitory factor did not reduce myofibre damage and no histological improvement was observed. The data presented here, while demonstrating a role for leukaemia inhibitory factor in myoblast proliferation, do not support a strong and consistent benefit of leukaemia inhibitory factor on dystrophic muscle *in vivo* as a means of alleviating the effects of chronic dystrophic muscle degeneration. © 2002 Elsevier Science B.V. All rights reserved.

**Keywords:** Leukaemia inhibitory factor; Skeletal muscle regeneration; Extracellular matrix

## 1. Introduction

Skeletal muscle has an excellent ability to regenerate and, in response to trauma, satellite cells (widely called myoblasts) become activated, proliferate, differentiate, and eventually fuse into myotubes that mature into myofibres [1]. Strategies to enhance regeneration are of clinical relevance in muscle disease such as Duchenne's muscular dystrophy (DMD) and after severe injury or transplantation of muscle tissue. Under certain circumstances, increased proliferation of myoblasts led to improved muscle regeneration [2,3]. To this end, it is of interest to test *in vivo* the influence of growth factors that are known mitogens for myoblasts. One such growth factor is leukaemia inhibitory factor (LIF).

There is a strong evidence for a role for LIF in myogenesis and data from Austin and colleagues suggest a potentially very important role for LIF in clinical treatment of myopathies and DMD. *In vivo*, LIF is upregulated in diseased and injured muscle [4,5] and the continual perfu-

sion of LIF from osmotic pumps into undamaged skeletal muscle of the dystrophic *mdx* mouse, or into crush injured skeletal muscle, enhances regeneration in terms of the number and the size of regenerated myofibres [4,6,7]. In tissue culture, LIF accelerates proliferation of both mouse and human myoblasts [8,9] and induces the formation of larger myotubes [10], although LIF has no effect on fibroblast cultures. Therefore, the *in vivo* action of LIF is probably related to specific myoblast proliferation with no effect on resident fibroblasts. The exogenous administration of basic fibroblast growth factor (bFGF) also increased replication of satellite and the proportion of myofibres showing evidence of regeneration (i.e. centralized nuclei) in the dystrophic *mdx* mouse [11]. The growth factors such as insulin-like growth factor-1 (IGF-1), bFGF and to a lesser extent nerve growth factor (NGF) also improve the histology of skeletal muscle regenerating after laceration injury [12]. In contrast with LIF and bFGF, other growth factors which stimulate myoblast proliferation *in vitro*, such as hepatocyte growth factor [13], interleukin 6 and transforming growth factor  $\alpha$  [7] are either ineffective or detrimental *in vivo* when applied to injured muscle [14].

The exact mechanism by which LIF enhances skeletal

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muscle regeneration is not clear. The present study specifically tests the effect of LIF on (i) myoblast proliferation, (ii) myofibre size, and (iii) myofibre resistance to damage. To test whether the reported increase in the number and size of myotubes *in vivo* is indeed a result of increased myoblast numbers, autoradiography [15] is used to quantify the effect of LIF on myoblast proliferation in the regenerating diaphragm of dystrophic *mdx* mice.

The *mdx* mouse is an animal model for the lethal X-linked myopathy DMD where dystrophic muscles undergo repeated cycles of endogenous breakdown and regeneration [16]. In humans with DMD, muscle regeneration eventually fails and death results, whereas in the *mdx* mouse, muscle regeneration is generally successful and skeletal muscle function is preserved [17]. After this, significant degenerative changes are observed in the limb muscles of *mdx* mice only after eccentric exercise [18,19]. However, in contrast to unexercised limb muscles, the diaphragm muscle of *mdx* mice does exhibit severe and long-term degeneration that begins before 3 months of age and progresses over the life of the mouse [20]. The diaphragm muscle more closely resembles the pathology of human DMD and therefore provides a suitable model for testing the efficacy of therapeutic substances as potential treatments for DMD. Previous studies have examined the effect of LIF on the histology and strength of *mdx* diaphragm muscles and reported a slowdown in the process of degeneration, as diaphragms continuously exposed to LIF contain more normal (peripherally nucleated) myofibres, larger regenerated fibres and less adipose tissue [21]. The amount of non-muscle (connective and adipose tissues) was significantly reduced and the maximum force producing the capacity of isolated diaphragm muscle strips was higher in LIF-treated mice [21].

LIF upregulates laminin expression *in vitro* [22] and it has been proposed that the beneficial effects of LIF on dystrophic muscle may (at least in part) be due to a strengthening of the myofibre, so that they are more resistant to damage and consequent necrosis (Austin personal communication). A loss of myofibre integrity is observed in  $\alpha 7$  integrin knockout mice [23] and myofibre integrity is enhanced after deflazacort administration [24]. Thus, agents that upregulate basement membrane components may be beneficial in ameliorating dystrophic progression. The present study is a direct extension of the previous work by Austin et al. [21]. It specifically tests the extent of myofibre damage in LIF treated-*mdx* muscles by using Evans Blue Dye (EBD) as a marker for myofibre integrity [25–28].

The sustained delivery of LIF *in vivo* was achieved by using alginate rods containing LIF [21,29] attached to the underside of the diaphragm of *mdx* mice. After 3 months, these LIF-treated dystrophic muscles were analysed autoradiographically and with EBD, to specifically address the impact of LIF on myoblast proliferation and myofibre integrity *in vivo*.

## 2. Materials and methods

### 2.1. Animals

All animal experiments were conducted in strict accordance with the guidelines of the University of Western Australia Animal Ethics Committee. Dystrophic *mdx* and C57BL10/ScSn (the parental strain for *mdx*) mice aged 12 weeks old were obtained from the Animal Resource Centre, Murdoch, Australia. Mice were housed in individual cages under a 12 h day/night cycle and allowed access to food and water *ad libitum*.

### 2.2. Delivery of LIF *in vivo*

Recombinant mouse LIF was kindly provided by AMRAD Corporation, Melbourne. Continuous delivery of LIF to the *mdx* mouse diaphragm was achieved via a rod of calcium alginate containing LIF. Each bio-compatible slow release alginate rod contained 1  $\mu\text{g}$  of LIF and released 5 ng of LIF per day per 15 mm (i.e. about 0.5% per day [29]).

### 2.3. LIF and regeneration in the *mdx* diaphragm

LIF releasing alginate rods were cut to 15 mm length, and sutured to the undersurface of the right hemi-diaphragm of four *mdx* mice at 12 weeks of age with a light retraction of the liver as described previously [21]. One LIF-treated *mdx* mouse died before the end of the experiment. Age-matched unoperated control *mdx* and C57BL10ScSn mice (three each) were included in the study. Unoperated *mdx* controls were used, as previous study [21] showed no difference between unoperated and saline-rod-treated diaphragms. At 12 weeks after implantation of LIF rods (and 10 days before sampling), each mouse received a single intra-peritoneal injection of  $^3\text{H}$ -thymidine ( $^3\text{H}$ -Tdr; Amersham) at 1  $\mu\text{Ci/g}$  body weight [30]. At 8 days after the  $^3\text{H}$ -Tdr injection (and 2 days before sampling) all the mice received a single intra-peritoneal injection of 1% w/v EBD (Sigma) at 0.1 ml/10 g body weight [28]. Two days later, all diaphragms were sampled (from the 6-month-old mice). The right hemi-diaphragm with the rod attached was divided into three equal parts (Fig. 1); one was fixed in 4% paraformaldehyde and processed for paraffin embedding, one was fixed in buffered formal saline and processed for resin embedding and autoradiography and morphometric analysis and the final sample was snap frozen in liquid nitrogen quenched isopentane for analysis of EBD labelling of myofibre damage.

### 2.4. Autoradiographic analysis of myoblast proliferation

It has been shown previously in the detailed studies that a period of 10 days allows sufficient time for the replicating myoblasts that had incorporated the  $^3\text{H}$ -Tdr (within 30 min), to cease proliferation and to fuse into multi-nucleated myotubes [30]: a sampling time of 10 days also eliminates

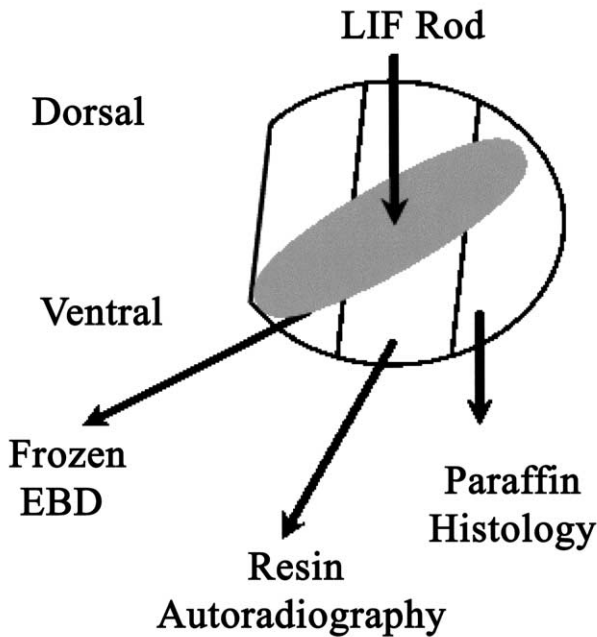


Fig. 1. Schematic representation of the positioning of the slow release LIF alginate rod on the right hemi-diaphragm and the sampling procedure. Each treated half of the diaphragm was divided into three parts as shown.

the possibility of subsequent necrosis of recently formed *mdx* myofibres [15]. Labelled centrally located muscle nuclei can be readily identified as myogenic. This approach allows retrospective analysis of the pattern of myoblast replication at the time when  $^3\text{H-Tdr}$  had been injected. Autoradiography on resin embedded muscle sections was performed essentially as described in detail previously [31]. In brief, all tissues were post-fixed in 1%  $\text{OsO}_4$  in 0.1 M phosphate buffer for 60 min washed in 70% ethanol and block stained in 1% *p*-phenylenediamine in 70% ethanol for 60 min. Tissues were infiltrated and embedded in a 1:1 Araldite/Epon mixture and 1  $\mu\text{m}$  sections cut for autoradiography. A few sections were coated with Kodak dipping emulsion and exposed in the dark in light tight boxes at 4°C for 2 weeks. Sections were then developed in Kodak D19, fixed in acid-hardener fixer, washed and air dried. Sections were viewed under a Leica DMLS light microscope with a 100 $\times$  oil immersion lens. In each section, at least 500 myogenic nuclei were counted and the number of centralized muscle nuclei labelled with three or more autoradiographic grains (Fig. 2(a)) were expressed as a percentage of the total number of muscle nuclei. This conservative labelling level of three grains has been used previously to allow for an adequate margin of error given a background labelling of 0.05 grains per 10  $\mu\text{m}^2$  [31].

### 2.5. Morphometric analysis of regeneration in the *mdx* diaphragm

Single transverse 5  $\mu\text{m}$  sections were taken from each

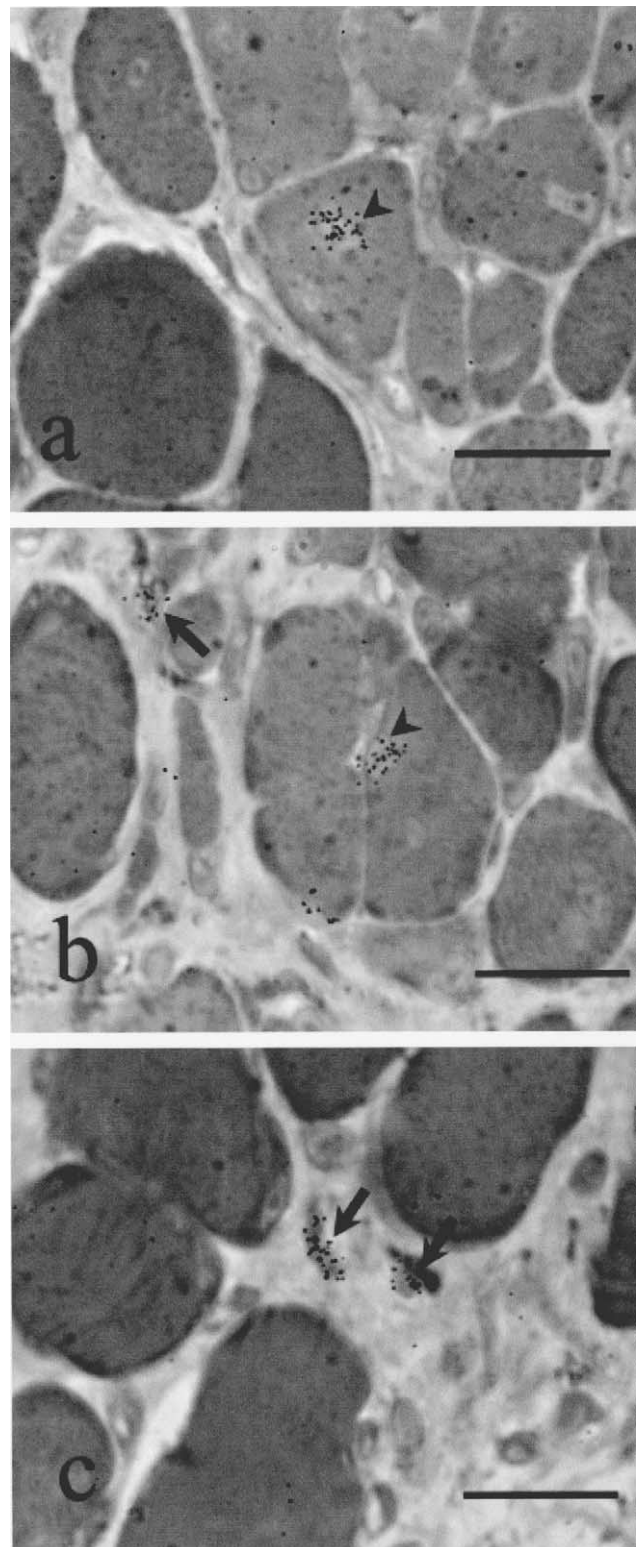


Fig. 2. Autoradiographic labelling of myotube nuclei in the *mdx* diaphragm where replicating cells had been labelled with  $^3\text{H}$ -thymidine at 12 weeks after grafting and muscles sampled 10 days later. The silver grains in the photographic emulsion are shown (in focus) overlying labelled (a) centrally and (b) peripherally located myotube nuclei (arrows), and (c) interstitial non-myogenic nuclei (arrows in (b) and (c)). Bar = 20  $\mu\text{m}$ .

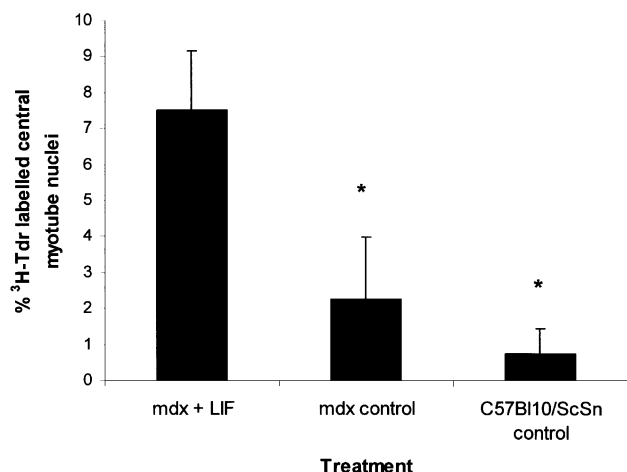


Fig. 3. Autoradiographic analysis of labelled myotube nuclei in the *mdx* and normal C57BL10/ScSn diaphragms. Histogram showing proportions (%) of labelled central myotube nuclei in LIF-treated and control (no LIF) *mdx* diaphragms of mice aged 6 months. The *mdx* diaphragms treated with LIF for 12 weeks show significantly more labelled myotube nuclei compared to diaphragms in age-matched unoperated *mdx* and normal C57BL10/ScSn mice (\* $P < 0.05$ ).

paraffin embedded sample at four different levels separated by 100  $\mu\text{m}$  and stained with haematoxylin and eosin (H&E). Sequential non-overlapping images of the entire area of each section were taken using Stage Pro computer software and a Hitachi HV-C20M 3CCD camera. The cross-sectional area of fibres with centralized and peripheral nuclei (centralized nuclei indicate that the myofibre has undergone regeneration) of each image was performed using Image Pro Plus 4.0. A minimum of 2000 fibres were analysed in each sample.

#### 2.6. EBD analysis of muscle fibre damage in *mdx* diaphragms

EBD was used as a marker for damaged muscle fibres in the *mdx* diaphragm, as it is bound to serum albumin and

readily enters through damaged or 'leaky' cell membranes into the sarcoplasm of myofibres. The presence of EBD in damaged myofibres is readily identified due to its red auto-fluorescence in tissue sections examined under fluorescence microscopy as has been described previously [25–28]. Three transverse sections (8  $\mu\text{m}$ ) separated by at least 150  $\mu\text{m}$  were taken from each frozen diaphragm sample. Sections were fixed in 70% ethanol, dehydrated, cleared in xylene and mounted using DPX. Sections were examined using fluorescence microscopy and the total number of EBD positive fibres in each of the three transverse sections was counted. The total number of myofibres in each section was counted using Nomarski microscopy. The number of EBD positive myofibres was expressed as a percentage of the total number of fibres for the muscle sample.

#### 2.7. Statistical analysis

Data are presented as mean  $\pm$  standard error (SEM) with the number of muscle fibres examined as  $n$ . Experimental groups have been statistically analysed using two-way analysis of variance (ANOVA) and Tukey–Kramer test and  $P < 0.05$  was considered significant. Analysis of fibre size distributions was performed using a two-sample Kolmogorov–Smirnov rank test.

### 3. Results

#### 3.1. Myoblast proliferation in *mdx* diaphragm muscle

The autoradiographic analysis of myoblast proliferation as measured by labelled myotube nuclei (Fig. 2) in *mdx* and control diaphragms is summarized in Fig. 3 and Table 1. Over 500 myofibre nuclei were examined in each diaphragm. The labelling of centrally located muscle nuclei (Fig. 2(a)) was sporadic, although myofibres with centrally labelled nuclei tended to occur in clusters and there was considerable biological variation. In all sections examined,

Table 1

Histological analysis of myofibre cross-sectional area, myoblast proliferation, EBD positive fibres and the extent of central muscle nucleation in LIF-treated *mdx* and control diaphragms at 6 months of age<sup>a</sup>

		<i>mdx</i> + LIF		<i>mdx</i> control		C57BL10/ScSn	
		Mean	( $\pm$ SEM)	Mean	( $\pm$ SEM)	Mean	( $\pm$ SEM)
Myofibres with peripheral nuclei	Mean size ( $\mu\text{m}^2$ )	486.9	(5.55)	519.6	(18.4)	582	(128)
	Cross-sectional area						
	Proportion (%)	36.8	(13.0)	53.8	(5.0)	96.9	(3.7)
Myofibres with central nuclei	Mean size ( $\mu\text{m}^2$ )	676.6	(21.8)	642.4	(37.1)	601	(95.4)
	Cross-sectional area						
	Proportion (%)	63.2	(13.0)	47.2	(2.9)	3.1	(3.7)
Labelled centralized nuclei	% total myonuclei	7.52	(1.65)	2.25	(1.72)	0.724	(0.7)
Evans blue dye positive fibres	% total fibre number	0.22	(0.11)	0.46	(0.05)	0.00	(0.00)

<sup>a</sup> A minimum of 2000 myofibres was measured in each diaphragm. The proportion of myofibres with central nuclei (indicating at least one cycle of regeneration), peripheral nuclei or EBD positive is expressed as a percentage (%) of the total population of myofibres. A minimum of 500 myonuclei was examined in each diaphragm and the percentage of labelled centralized nuclei (equating to proliferating myoblasts at the time of injection) is expressed as a percentage (%) of total myonuclei counted. All values are the average of three mice ( $\pm$ SEM).

the level of labelled peripheral myonuclei (Fig. 2(b)) was consistently low and was not statistically different when comparing LIF-treated and any control *mdx* diaphragms. In all *mdx* diaphragms, a significant number of nuclei in the interstitial space were labelled (Fig. 2(c)), although a detailed analysis was not undertaken. In control (non-dystrophic) C57BL/10ScSn diaphragms, the level of post-mitotic labelling of any nuclei was extremely low. In LIF-treated *mdx* diaphragms, the number of labelled central nuclei was significantly higher ( $P < 0.05$ ) than in age-matched untreated *mdx* and normal C57BL/10ScSn diaphragms. The increased number of labelled muscle nuclei in LIF-treated *mdx* diaphragms was also statistically significant ( $P < 0.05$ ) when compared to normal (non-dystrophic) C57BL/10 diaphragms. There was no statistical difference between unoperated *mdx* and C57BL/10/ScSn control muscles.

### 3.2. Histological analysis of the effect of LIF on the *mdx* diaphragm

After 12 weeks of LIF infusion from slow release alginate gels, the effect of LIF on myofibre cross-sectional areas and the proportion of myofibres with central nuclei was examined in the *mdx* diaphragm (Fig. 4). The data from the morphometric analysis of myofibre size are summarized in Table 1. The effect of LIF on *mdx* diaphragm muscle was compared with untreated age-matched *mdx* mouse diaphragms and with normal C57BL/10ScSn diaphragms. The cross-sectional area was considered in three categories of fibres within the diaphragms including (i) myofibres with centralized nuclei (regenerated), (ii) myofibres with only peripheral nuclei and (iii) all myofibres. No significant differences between the cross-sectional areas of the myofibres (with centralized or peripheral nuclei) were observed for LIF-treated and untreated control (no LIF) *mdx* diaphragms (Table 1). The variation in myofibre size was far greater in control (no LIF) diaphragms compared to both LIF treated *mdx* and normal C57BL/10ScSn diaphragms. The size distribution of myofibres with both centrally (Fig. 5(a)) and peripherally (Fig. 5(b)) located nuclei was also determined. The size distribution of these two groups of myofibres did not differ significantly between LIF treated *mdx* and control untreated *mdx* diaphragms. The proportion of myofibres with central nuclei was also similar in LIF-treated and untreated *mdx* diaphragms. As expected, in the normal control C57BL/10/ScSn muscles, very few myofibres had central nuclei and the sizes shown in Table 1 are derived from only 1500 myofibres.

### 3.3. The effect of LIF on myofibre integrity in the *mdx* diaphragm

EBD was used as a marker for myofibre damage; the data are summarized in Table 1. At sampling (48 h after EBD injection) macroscopically, there was a high level of non-specific (blue) colouration of the diaphragm, abdominal

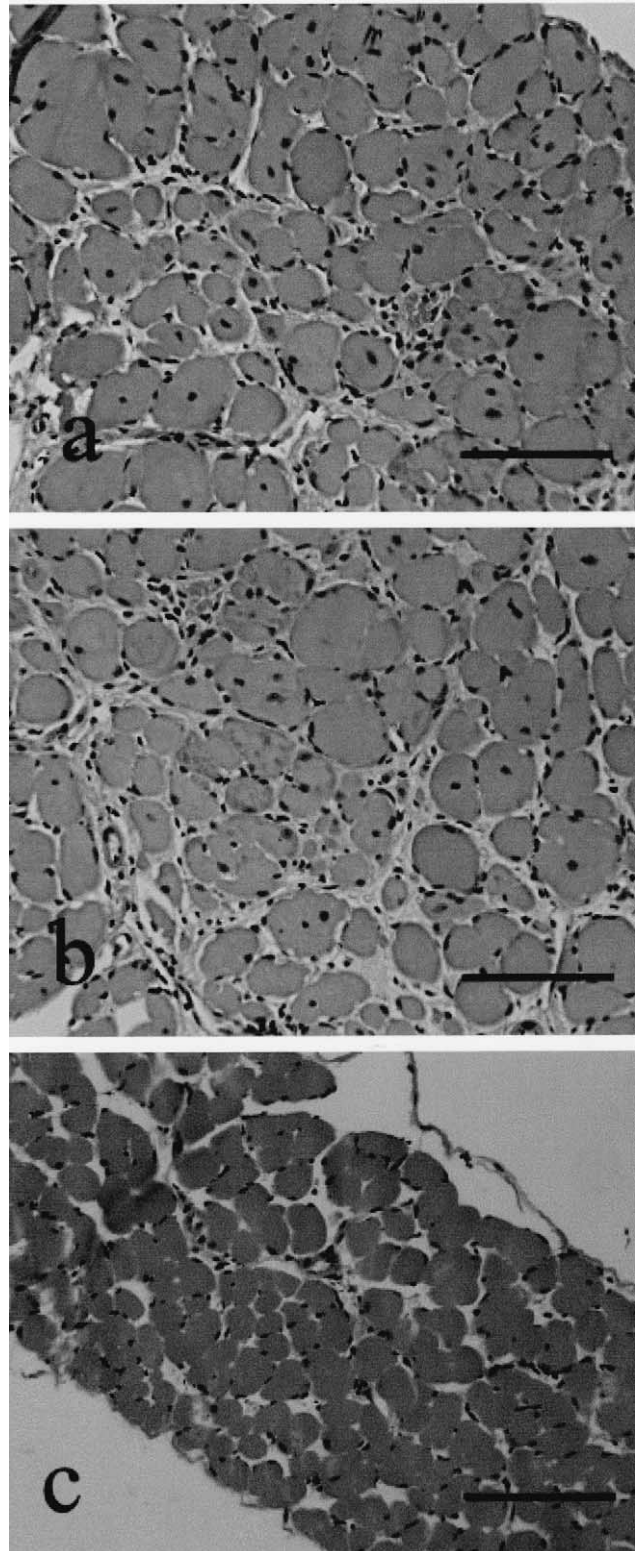


Fig. 4. Histology of (a), (b) *mdx* and (c) normal C57BL/10/ScSn diaphragms at 6 months of age. H&E staining of (a) LIF-treated *mdx* and (b) untreated control *mdx* diaphragms shows evidence of the ongoing myopathy with numerous myofibres with centralized nuclei (these fibres have regenerated) throughout the tissue and a wide variation in myofibre cross-sectional area. This contrasts with the (c) normal appearance of (non-dystrophic) C57BL/10/ScSn diaphragm myofibres. Bar = 100  $\mu\text{m}$ .

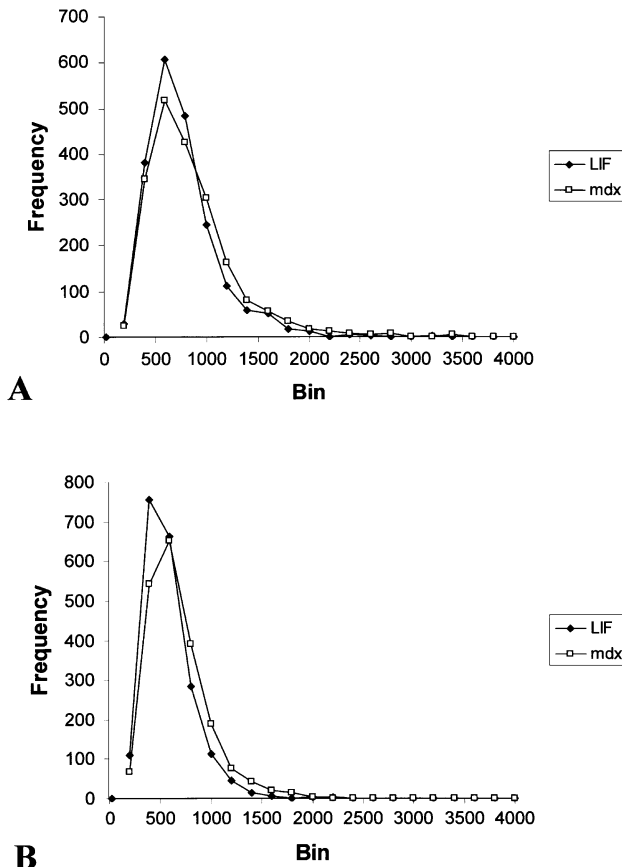


Fig. 5. Frequency histogram (bin width =  $200 \mu\text{m}^2$ ) depicting the distribution of fibre sizes in LIF treated and unoperated *mdx* diaphragms. The size distribution of myofibres with (a) central and (b) peripheral nuclei was not different between LIF-treated (solid line) and control untreated (dotted line) *mdx* diaphragms.

muscles and organs. This was not evident above the diaphragm in the thoracic cavity. Furthermore, microscopically, the most intense staining of EBD-treated diaphragms was associated with blood vessels. The number of EBD positive fibres in each diaphragm was expressed as a percentage of the total number of fibres. None of the myofibres were labelled with EBD in normal non-dystrophic C57Bl/10ScSn (emphasizing the healthy nature of this muscle). The proportion of EBD-positive myofibres in dystrophic *mdx* diaphragms was significantly higher (0.22 and 0.46%) than in normal (non-dystrophic) C57Bl/10ScSn diaphragms. While the number of EBD positive myofibres appeared to be lower in LIF-treated *mdx* diaphragms (0.22%) compared with untreated *mdx* muscle (0.46%), there was no statistical difference. In longitudinal section, many of the EBD-positive myofibres in *mdx* muscles also appeared to be necrotic.

#### 4. Discussion

Unlike skeletal muscles of the limbs in *mdx* mice where

the dystrophic process abates after an initial acute episode around 3–4 weeks of age [32,33], the diaphragm shows progressive degeneration and severe pathology over time [20]. Previous studies by Austin et al. [21] reported an improved histology (increased myofibre cross-sectional area and reduced interstitial tissue) in *mdx* diaphragms at 6 months of age associated with an increase in force generation in response to LIF treatment for 12 weeks. They suggested that this was the direct result of increased myoblast proliferation (based on *in vitro* studies), but could also be due to increased myofibre size and resistance to damage. The present study specifically examined the effects of LIF on (i) myoblast proliferation, (ii) myofibre size and (iii) myofibre damage in the *mdx* diaphragm.

The data presented here suggest that myoblast replication increased significantly in dystrophic muscle *in vivo* by LIF. This is consistent with a previous autoradiographic study using crush injured normal muscle where the number of replicating myoblasts in LIF-treated crush injured muscle was maintained at a higher level than in control crush injured muscles [22]. The increased number of labelled myonuclei could be the result of (i) a larger population of myoblasts being labelled at the time of injection, (ii) the same number of myoblasts being labelled but surviving longer before fusion, or (iii) labelled myoblasts undergoing extra cell divisions before fusion. While extra cell divisions can result in delayed fusion, previous *in vitro* studies do not provide evidence for delayed fusion in LIF-treated myoblast cultures [10,34]. Thus, it seems unlikely that this accounts for the present observations. The most likely scenarios to account for the increased labelling are a larger population of replicating myoblasts at the time of  $^3\text{H.Tdr}$  injection or an increase in subsequent myoblast survival. The latter is supported by *in vitro* evidence showing that LIF increases myoblast survival [22].

In contrast to a reported beneficial effect of LIF on *mdx* diaphragm histology [21], we did not see the evidence of myofibre hypertrophy with LIF. The two sets of experiments were conducted on *mdx* mice of the same age but from colonies that had been separated for many years (one in Melbourne, Victoria and the other in Perth, Western Australia). Some significant differences in the pathology of control (unoperated) *mdx* diaphragms from the two studies were apparent with the mean area of myofibres with centralized nuclei in the diaphragm being significantly higher in the control *mdx* mice in Perth ( $787 \mu\text{m}^2$  versus  $380 \mu\text{m}^2$ , respectively). In contrast, the mean area of myofibres with centralized nuclei is quite similar in the test groups of LIF-treated *mdx* diaphragms between the two studies ( $583 \mu\text{m}^2$  compared to  $570 \mu\text{m}^2$ , respectively). The difference between the sizes of myofibres in untreated control *mdx* mice clearly affects the significance of the data obtained with LIF treatment. It should be noted that the Perth data are based on three mice and 500 myofibres compared to six mice and 4000 myofibres in the study by Austin et al. [21], but this difference in the size of the studies is not likely to

obscure a major beneficial effect of LIF. It is crucial that claims of therapeutic benefits of agents on DMD are carefully evaluated by different groups in order to substantiate benefits for potential clinical applications [35].

Any technical differences in the delivery of LIF *in vivo* were minimized between the studies by using LIF and alginate from the same source. That the delivery of LIF via these alginate gels is effective in the present study is confirmed by data from a parallel study where increased myoblast proliferation was seen in LIF-treated crush injured skeletal muscle [22]. However, in the same study, it can be observed that LIF did not affect myotube size or the composition of the extracellular matrix at 7 days in muscle regenerating after crush injury. This lack of effect of LIF on myotube size corresponds with a recent report on rat skeletal muscle regenerating after bupivacaine injection where LIF, delivered systemically, had no effect on functional capacity or the size of regenerated myofibres [36]. However, LIF may be effective when administered with other agents because, while LIF alone does not affect skeletal muscle fibre hypertrophy in rats, when administered in combination with clenbuterol, a significant increase in myofibre diameter is observed compared to both LIF and clenbuterol alone [37]. Our studies with EBD also showed that LIF did not make a significant difference to the percentage of myofibres that are damaged compared with age-matched untreated *mdx* controls. The numbers of EBD positive fibres (in the *mdx* at 24 weeks of age) reported here are consistent with previous studies using EBD to examine myofibre integrity in the *mdx* diaphragm. In sedentary (unexercised) 2 months old *mdx* mice, Brussee et al. [19] report that EBD positive fibres are rare in the diaphragm, although the number of EBD positive fibres does increase with exercise.

Overall, our combined data provide no evidence that sustained exposure to LIF has a beneficial effect on the dystrophic process (myofibre size and susceptibility to damage) *in vivo* and they do not support the proposal that LIF (alone) might be used as a therapeutic agent to ameliorate or minimize the pathologic affects of muscular dystrophy.

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