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REGULAR ARTICLE

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Leukaemia inhibitory factor increases myoblast replication and survival and affects extracellular matrix production: combined in vivo and in vitro studies in post-natal skeletal muscle

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Abstract Leukaemia inhibitory factor (LIF) has been reported to specifically enhance myoblast proliferation in vitro and increase the number and size of myotubes in regenerating skeletal muscle in vivo. The present study specifically tests the effect of LIF on myoblast replication in vivo. Administration of exogenous LIF by slow release alginate gels in vivo sustained the level of myoblast proliferation at 2 days in regenerating crush-injured muscle. Since the extracellular matrix (ECM) plays an important role in regulating the effects of many growth factors, the hypothesis was tested, both in vivo and in vitro, that some of the beneficial effects of LIF are mediated by modulation of the ECM. The effects of LIF in vivo on the amount and localisation of the ECM molecules, fibronectin, tenascin-C, collagen type IV and laminin were assessed by immunohistochemistry on regenerating skeletal muscle but no influence of LIF on ECM composition was observed. In tissue culture, LIF increased BALB/c myoblast proliferation at day 3 on culture dishes coated with Matrigel and also increased the viability in vitro of BALB/c myoblasts grown under suboptimal conditions. Quantitation of the ECM produced by cultures (enzyme-linked immunosorbent assay) showed that LIF affected the amount of fibronectin, tenascin-C, collagen type IV and laminin produced by fusing myoblasts. No significant affect of LIF was seen on myotube formation either in vitro or in vivo. These combined in vitro and in vivo studies show an effect of LIF on ECM production in vitro, on myoblast survival and on in vivo myoblast replication.

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Introduction

The excellent regeneration of post-natal skeletal muscle results from the activation of muscle precursor cells, which are normally present as quiescent satellite cells, beneath the basement membrane of myofibres. Activated satellite cells, widely called myoblasts, then proliferate, differentiate and eventually fuse into myotubes that mature into myofibres (Grounds 1991). Skeletal muscle regeneration is regulated by growth factors and these interact with components of the extracellular matrix (ECM; Martins-Green 2000). Growth factors that appear to be of key importance in muscle regeneration include leukaemia inhibitor factor (LIF), insulin like growth factors I and II, fibroblast growth factors (FGF) 1 and 6, platelet-derived growth factor and hepatocyte growth factor (Kurek et al. 1998; Grounds 1999; Miller et al. 2000). This study focuses on the actions of LIF, which has been shown to enhance several aspects of new muscle formation both in vitro and in vivo.

LIF stimulates the proliferation of myoblasts (but not fibroblasts) in vitro, although the response to LIF appears to differ from other mitogens because of an extended lag period (Austin and Burgess 1991; Austin et al. 1992). In addition to the mitogenic action, it is reported that LIF enhances myotube formation and that larger myotubes are formed in vitro (Vakakis et al. 1995). LIF is an autocrine survival factor for Schwann cells (Dowsing et al. 1999), is associated with the survival of skin allografts (Akita et al. 2000) and is considered to be anti-apoptotic (Fukada et al. 1996). Furthermore, in vivo, LIF is up-regulated in diseased and injured muscle (Barnard et al. 1994; Kurek et al. 1996a) and after sciatic nerve transection (Kurek et al. 1996a). Exogenous LIF enhances skeletal muscle regeneration after crush injury and in the *mdx* mouse model of Duchenne muscular dys-

trophy (Kurek et al. 1996b, 1997) and the regeneration of transected peripheral nerve (Tham et al. 1997). LIF slows the rate of degeneration in *mdx* diaphragm; it has been suggested that this may be the result of a strengthening of the cell membrane around myofibres and interaction with adjacent ECM (Austin et al. 2000). It is not clear whether the enhanced skeletal muscles regeneration reported with LIF is attributable mainly to increased myoblast proliferation or to superior myotube formation. Therefore, the effect of LIF on myoblast proliferation is specifically assessed by using autoradiography in muscles of BALB/c mice regenerating after crush injury in the present study.

ECM remodelling (Kherif et al. 1999) together with myoblast migration, proliferation and fusion are essential aspects of skeletal muscle formation in vivo during regeneration. The ECM modulates many aspects of cell behaviour during tissue formation in development and regeneration (Martins-Green 2000). Since many diffusible factors have to cross the basement membrane before reaching the cell surface receptors, this interaction may be proceeded by intermediate binding to components of the ECM. For example, cell-surface heparin-sulphate proteoglycans bind to FGF-1, acting as a reservoir and thus changing the half-life and increasing the cellular response. LIF in vivo is localised to the ECM via interaction with a 140-kDa ECM-localised binding protein (Mereau et al. 1993) suggesting that the mechanism for LIF is similar to the regulation of bFGF by the ECM. In contrast to the apparent lack of importance of ECM on mitogenic effects of growth factors (Schollmann et al. 1992; Maley et al. 1995), there is strong evidence that the nature of the ECM substrate substantially influences myotube formation (Maley et al. 1995). Therefore, the pronounced effect of LIF on myotube formation might be mediated (in part) by modification of the ECM environment.

Whereas changes in ECM expression during regeneration have been described (Dodson et al. 1996; Grounds et al. 1998; Sorokin et al. 2000), the influence of exogenous growth factors, such as LIF, on ECM production has not been examined. The present study tests the effects of combinations of LIF and various ECM substrates on myoblast proliferation and also the influence of LIF on ECM production by myogenic cells of BALB/c mice in vitro. We have also investigated, in vivo, possible alterations in ECM composition in response to LIF, at various times after in crush injury to skeletal muscle of BALB/c mice. Furthermore, immunohistochemistry with a wide range of antibodies has been used to examined ECM components found in the interstitial connective tissue, viz. fibronectin and tenascin-C, and ECM molecules closely associated with the basement membrane of myofibres, viz. laminin and collagen type IV (Grounds et al. 1998; Ringlemann et al. 1999; Sorokin et al. 2000). These combined studies on skeletal muscle have been designed to provide precise information on the mechanism of action of LIF in vivo.

Materials and methods

Animals

Experiments were conducted in strict accordance with the guidelines set out by the Animal Ethics Committee of The University of Western Australia. Inbred BALB/c mice aged 4–6 weeks were obtained from the Animal Resource Centre, Murdoch, Western Australia, housed in individual cages under a 12-h day/night cycle and allowed access to food and water ad libitum.

In vivo studies

Surgical procedure for crush injury

The mid section of both tibialis anterior (TA) muscles of 36 BALB/c mice was crushed in a standarised manner (Mitchell et al. 1992). Briefly, anaesthesia was achieved with inhaled Halothane (1.5%–2%) delivered in O_2, both legs were shaved and a longitudinal incision in the skin was made over the TA. At the mid belly of the TA, the muscle was dissected free of the tibia, taking care not to disturb either the nervous or blood supply to the tissue. One arm of a pair of artery forceps was inserted between the TA and tibia and a 5-s crush injury was delivered to the muscle. The resulting lesion was approximately 4 mm wide in all cases. The skin was closed with a 6–0 braided silk suture.

In vivo delivery of LIF

Recombinant mouse LIF was kindly provided by AMRAD, Melbourne. Alginate rods containing LIF were prepared as described previously (Austin et al. 1997). The rods were cut to 15 mm lengths and implanted subcutaneously immediately adjacent to the TA on the outside of the leg. In all cases, the rods were implanted at the time of surgery and were left in place throughout the experimental period. Each LIF rod contained 1 µg LIF and the release rate was approximately 5 ng LIF per day, based on previous data (Austin et al. 1997).

Autoradiographic analysis of cell proliferation

LIF-treated and controls groups each consisted of four muscles (from two mice). At 3, 5 and 7 days after surgery, four mice (two treated with LIF and two controls) received a single intra-peritoneal injection of ^{3}H-thymidine (1 µCi/g body weight). The crush injured TA muscles from each mouse were sampled 10 days after injection, fixed in buffered formalin saline and processed for resin embedding and autoradiography (see below).

The sampling of muscles at 10 days after ^{3}H-thymidine injection was designed to allow time for the replicating myoblasts that had incorporated ^{3}H-thymidine (within 30 min) to cease proliferation and to fuse into multi-nucleated myotubes, where the labelled centrally located muscle nuclei could be readily identified as myogenic. This approach allowed retrospective analysis of the pattern of myoblast replication at the time when ^{3}H-thymidine had been injected. Autoradiography on resin-embedded muscle sections was performed essentially as described previously (Grounds and McGeachie 1989; Roberts et al. 1989). In brief, all tissues were post-fixed in 1% OsO_4 in 0.1 M phosphate buffer for 60 min, washed in 70% ethanol and block-stained in 1% para-phenylenediamine in 70% ethanol for 60 min. Tissues were infiltrated and embedded in a 1:1 Araldite/Epon mixture and 1 µm sections were cut for autoradiography. Sections were coated with Kodak dipping emulsion and exposed in the dark in light tight boxes at 4°C for 2 weeks. Sections were 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× oil immersion lens. In each section, at least 500 myogenic nuclei were counted and the number of centralised muscle

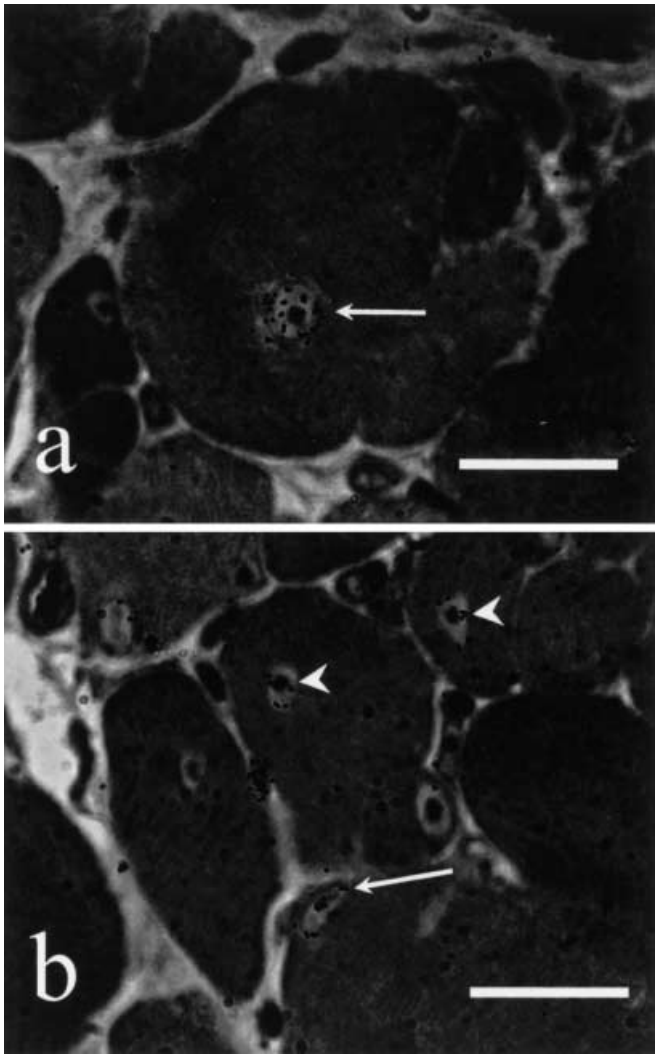


Fig. 1a, b Autoradiographic labelling of myotube nuclei in a regenerating crush-injured BALB/c muscle in which myoblasts were labelled with ^{3}H-thymidine at 5 days after injury and sampled 10 days later. The silver grains in the photographic emulsion are shown (in focus) overlying labelled centrally (**a**) or peripherally (**b**) located myotube nuclei (*arrows*). Interstitial non-myogenic nuclei are often labelled, especially in the central core of the crush lesion. *Bar* 20 µm

nuclei and peripheral muscle nuclei (assumed to correspond to satellite cells) labelled with three or more autoradiographic grains (Fig. 1a) were expressed as a percentage of the total number of muscle nuclei. This conservative labelling level of three grains was used previously to allow for an adequate margin of error given a background labelling of 0.05 grains per 10 µm² (Grounds and McGeachie 1989).

In vivo production of ECM molecules

At 3, 5, 7 and 10 days after crush injury and implantation of the rods, six muscles (from three mice) were sampled from LIF-treated and control mice. The injured TA muscles from both legs were removed and either frozen immediately in liquid-nitrogen-cooled isopentane (four samples) or immersion-fixed in 4% paraformaldehyde (two samples). Frozen tissues were stored at –80°C until required.

For frozen muscles, longitudinal sections were cut at a thickness of 8 µm on a Leica cryomicrotome and placed onto silanated glass slides. Paraformaldehyde-fixed samples were processed for paraffin embedding and 5 µm transverse sections were cut and stained with haematoxylin and eosin for routine microscopy and morphometric analysis.

Immunohistochemistry

Details of the antibodies used to stain the various ECM molecules are outlined in Table 1. In addition, antibodies to the cytoskeletal protein desmin were used to identify myoblasts and myotubes within the crush lesion (White et al. 2000). The primary antibodies were detected by using either Cy3-conjugated or ALEXA-546-conjugated anti-rabbit or anti-rat secondary antibodies (see Table 1).

Frozen sections were allowed to equilibrate to room temperature before being rehydrated in phosphate-buffered saline (PBS) for 20 min. Non-specific binding was blocked with 5% fetal calf serum (FCS), PBS, 0.1% glycine, 0.1% bovine serum albumin (BSA) for 60 min at room temperature. Primary antibodies were diluted in wash buffer (0.5% BSA, 0.1% glycine, PBS) and incubated with the sections at room temperature for 60 min. After being washed in buffer, the bound primary antibody was detected by using secondary antibodies (diluted in wash buffer) at room temperature for 60 min. Nuclei were stained by means of Hoechst 33342 and sections were mounted in polyvinyl acetate.

Morphometric analysis of regeneration

A single transverse 5-µm-thick section was cut from each paraffin-embedded sample and stained with haematoxylin and eosin. Se-

Table 1 Details of primary and secondary antibodies used in immunohistochemistry

Specificity	Species/ primary source	Conjugate	Streptavidin	Dilution	Source
Primary antibodies					
Fibronectin	Rabbit	–	–	1:400	Sigma
Tenascin C	Rat	–	–	1:200	Sigma
Laminin	Rabbit	–	–	1:400	Sigma
Collagen IV	Rabbit	–	–	1:200	Biodesign
Desmin	Goat	–	–	1:75	Santa Cruz
Secondary antibodies					
	Rabbit	Cy3	N/A	1:500	Amersham
	Rat	Biotin (1:200)	ALEXA546	1:10,000	Molecular Probes
	Goat	ALEXA 488	N/A	1:1000	Molecular Probes

quential non-overlapping images of the entire area of each section were taken by using Stage Pro computer software and a Hitachi HV–C20 M 3CCD camera. Morphometric analysis of each image was performed by using Image Pro Plus 4.0. The morphometric parameters considered were the cross-sectional area of fibres with centralised and peripheral nuclei and the maximum and minimum area observed for this total population of myofibres. At least 500 myofibres were counted in each section.

In vitro studies

Establishment of primary myoblast cultures

Primary cultures of skeletal muscle were established essentially as described previously (Maley et al. 1994, 1995). In brief, the hindlimb and lower back muscles from eight BALB/c mice aged 4 weeks were removed and minced. Sequential enzymatic digestion with collagenase, dispase and trypsin was used to break down the tissue structure. The digestion mixture was washed and coarse-filtered to remove tissue debris. The resulting cells were seeded into flasks coated with 1% (v/v) gelatin in Ham's F10 supplemented with 20% (v/v) FCS and 25 ng/ml bFGF.

Myoblast proliferation in vitro

The effect of LIF on the proliferation of myoblasts grown on various culture substrates was evaluated by measuring the level of incorporation of tritiated thymidine essentially as described previously (Pavlath et al. 1998).

Cell culture

The surfaces of 24-well culture plates were either left uncoated or coated with gelatin (denatured collagen type I, 1% w/v; Sigma), fibronectin (50 µg/ml; Sigma), collagen type IV (50 µg/ml; Sigma) or a "thin layer" of Matrigel (Becton Dickison), as described by the manufacturer. Matrigel is a basement-membrane-like substrate composed mainly of laminin-1, collagen IV, proteoglycans, entactin and nidogen and some growth factors. Some wells were first coated with gelatin and then washed and fibronectin was bound to the gelatin layer, as the substrate that fibronectin binds to has been shown to affect myogenic C2C12 proliferation significantly (Garcia et al. 1999). Equal numbers (1×10^4) of BALB/c myoblasts in Ham's F10 supplemented with 5% FCS were plated into each well and LIF was added to half of the wells. In all the in vitro studies, LIF was used at 0.3 ng/ml, as previous studies have shown that this elicits an optimal response by myoblasts (Austin and Burgess 1991; Austin et al. 1992).

H^3-thymidine incorporation

After 3, 5, 7 and 10 days, the cells were pulsed with 1 µCi/ml [6–^{3}H] thymidine (Amersham; specific activity 24 Ci/mmol) for 2 h at 37°C. Excess [6–^{3}H] thymidine was washed from the wells with two changes of cold PBS. Cells were treated with cold 20% (w/v) trichloroacetic acid for 30 min at 37°C and lysed for 10 minutes with 0.1 M NaOH and 0.1% sodium dodecyl sulphate (SDS). Lysates were mixed with Emulsifier-Safe scintillation fluid (Packard) and counted in a Packard 1500 Tri-Carb liquid scintillation counter. Quadruplicate wells were assayed for each experimental condition.

Myoblast viability

Since the absolute number of cells in a culture can represent a balance between cell proliferation and cell death, the effect of LIF on myoblast viability was assessed in culture. Trypan blue dye (Sigma) exclusion was used to quantitate the viability of myoblast cultures. Equal (3×10^4) numbers of primary BALB/c myoblasts were seeded into gelatin-coated 24-well plates in Ham's F10 plus 5% (v/v) FCS. At time 0, half the wells were treated with 0.3 ng/ml LIF and the remaining wells were used as controls (no LIF). At 1, 3, 5, 7 and 10 days, all cellular material was removed from each well, including attached cells after trypsinisation and all cellular material floating free in supernatant, and pelleted. The pelleted cells were suspended 50 µl in Ham's F10 plus 20% FCS. A 20-µl aliquot was transferred to a sterile microfuge tube and diluted 1:5 with 0.4% (w/v) trypan blue in sterile saline. Viable cells had an intact membrane; they did not take up the dye and appeared clear by light microscopy. The total number of cells, both viable and not, in a defined volume (1×10^{-4} ml) was counted by using a haemocytometer and the number of viable cells was expressed as a percentage of the total number of cells.

Quantitation of ECM production

Myotube formation

A total of 1×10^4 primary BALB/c myoblast in Ham's F10 medium supplemented with 20% (v/v) FCS were plated into 96-well plates coated with 1% (w/v) gelatin and allowed to adhere. Half the wells were treated with 0.3 ng/ml LIF at the time of seeding. After 48 h, the medium was removed, the cultures were washed in PBS and "fusion medium" consisting of DMEM supplemented with 2% (v/v) horse serum was added to induce differentiation and fusion (0.3 ng/ml LIF was again added to wells at seeding). Time 0 was defined as the point at which fusion medium was added. The fusion media was replenished daily.

Enzyme-linked immunosorbent assay

The amount of fibronectin, tenascin-C, laminin and collagen type IV bound to the cultured cells was quantitated by using enzyme-linked immunosorbent assay (ELISA) directly on the cell layer as outlined below. Assays were performed in five separate wells derived from common source cultures (for antibodies and dilutions, see Table 2. After 4 and 7 days in fusion medium (as described above), the presence of myotubes was confirmed under phase microscopy, the excess medium was washed from the wells with PBS, and non-specific binding was blocked with 10% FCS, PBS, 1% BSA. Primary antibodies were diluted in PBS, 0.1% BSA and incubated with the cells for 60 min at 37°C. After a washing step in PBS, horseradish-peroxidase-conjugated secondary antibodies were diluted in PBS, 0.1% BSA and incubated with the cells for 60 min at 37°C. To detect the presence of bound secondary antibodies, 100 µl fresh o-phenylenediamine (Sigma) dissolved in methanol and tri-sodium citrate buffer (pH 4) was added and left for up to 15 min at room temperature. The yellow colour reaction was stopped with 50 µl 12.5% sulphuric acid. The optical density (OD) of each well was read at 490 nm. The OD for each well was then expressed as a ratio to the amount of DNA present (see extraction protocol below) to correct for any variation in cell numbers that may have occurred.

DNA extraction

After the ELISA procedure (see above), cells were lysed with 200 µl DNA extraction buffer (50 mM TRIS, 150 mM NaCl, 2 mM EDTA) and SDS was added to a final concentration of 0.5% (w/v). After incubation at 65°C for 20 min, proteinase K (200 µg/ml) was added and the mixture incubated at 37°C overnight. The digestion volume was brought to 500 µl with 10 mM TRIS-HCl, 1 mM EDTA (TE buffer) and the protein was extracted by using equilibrated phenol at pH 8. Contaminating phenol was removed from the aqueous phase with an equal volume of chloroform:iso-amyl alcohol (24:1). DNA was precipitated from the aqueous phase with two volumes of cold ethanol at –20°C over-

Table 2 Details of primary and secondary antibodies used in ELISA

Specificity	Species/ primary source	Conjugate	Dilution	Source
Primary antibodies				
Fibronectin	Rabbit	–	1:1000	Sigma
Tenascin C	Rat	–	1:500	Sigma
Laminin	Rabbit	–	1:100	Sigma
Collagen IV	Rabbit	–	1:500	Biodesign
Secondary antibodies				
	Rabbit	Peroxidase	1:2000	DAKO
	Rat	Peroxidase	1:1000	DAKO

night and the DNA pellet was resuspended in TE buffer and quantified by reading the OD at 260 nm.

Statistical analysis

In all cases, differences were compared between LIF-treated and control groups. The significance of any difference was analysed by using Student's *t*-test.

Results

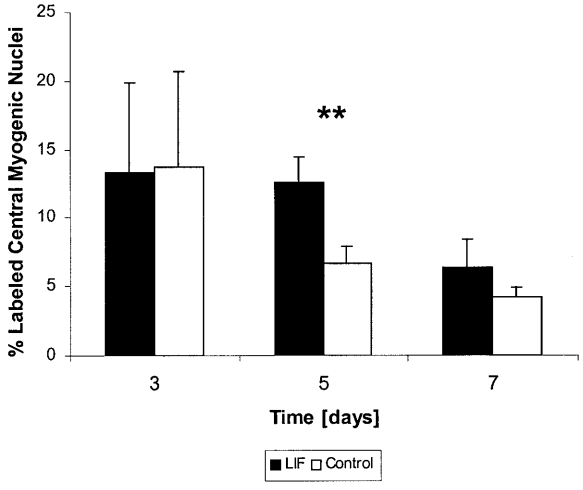
Effect of LIF in vivo after crush injury

Myoblast proliferation in crush-injured muscle

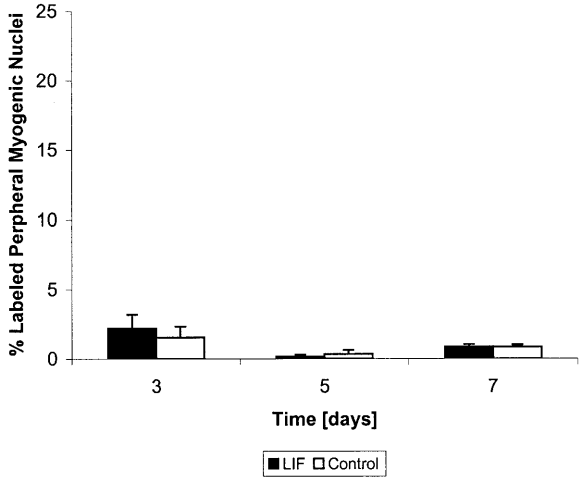
At 3 days after injury, the numbers of labelled centralised myotube nuclei (Fig. 1a) in crush-injured muscle were similar in LIF-treated and control muscles (Fig. 2a). At 5 days after injury, the number of labelled centralised myotube nuclei was significantly higher ($P<0.05$) in regenerating muscles exposed to exogenous LIF (Fig. 2a). At day 5, LIF maintained the level of labelling seen at day 3, whereas there was a decrease in myotube nuclear labelling (indicative of myoblasts that had been replicating at this time) in untreated muscle at 5 days after crush injury. At 7 days, the number of labelled centralised myotube nuclei had decreased in LIF-treated crush-injured muscles to a level comparable with that of the control muscle samples (Fig. 2a). The numbers of labelled nuclei in myotubes that were peripherally located (Fig. 1b) were not significantly different at any time between LIF-treated and untreated control muscle samples (Fig. 2b).

Histology of crush-injured muscle

In crush-injured BALB/c muscles, three distinct regions were noted including the central necrotic zone, the adjacent zone of regeneration where active myotube formation was occurring, and the surrounding undamaged muscle, as described by Mitchell et al. (1991). Morphometric analysis of muscles sampled 3 days after crush injury showed that the initial size of the lesions did not vary significantly between LIF-treated (i.e. sustained re-



a – Labeled central myotube nuclei



b – Labeled peripheral myotube nuclei

Fig. 2a, b Autoradiographic analysis of myotube nuclei in regenerating crush injured skeletal muscle. **a** Histogram showing proportions (%) of labelled centralised myotube nuclei in LIF-treated and control crushed muscle at 3, 5 and 7 days after surgery. At 5 days after surgery, there was a significant increase in the level of myoblast proliferation in LIF-treated muscles. **b** Histogram showing proportions (%) of labelled peripheral myotube nuclei in LIF-treated and control crushed muscle at 3, 5 and 7 days after surgery

Table 3 Morphometric analysis of myotube cross-sectional area (μm^2) in regenerating BALB/c skeletal muscle at 7 days after injury. Two TA muscles were analysed for each mouse and the data for each mouse are presented

	LIF (5 ng/day)		Control (untreated)	
	Animal 1	Animal 2	Animal 3	Animal 4
Mean fibre area (±SD)	443 (±207)	436 (±227)	450 (±225)	376 (±209)
Maximum fibre area	1452	1808	1800	1567
Minimum fibre area	127	102	89	76

lease of LIF from alginate rods) and control (untreated) muscles. No myotubes were seen at 3 days. Myotubes were present at day 5 and many well-developed myotubes were present at days 7 and 10. The morphometric analysis of the cross-sectional area of myotubes in the regenerating zone of crush-injured muscle after 7 days is summarised in Table 3. There was no significant difference in the mean, maximum and minimum cross-sectional area of myotubes between LIF-treated and control (no LIF) groups at day 5 (data not shown) or day 7 (Table 3).

Immunohistochemical staining and analysis

Immunohistochemical staining for fibronectin, tenascin-C, laminin and collagen type IV was examined in crush-injured BALB/c muscle at day 3 (early stages of regeneration), day 5 (mid stages of regeneration), day 7 (late regeneration) and day 10 (regeneration essentially complete). No striking differences in the staining pattern were noted for any of the ECM molecules in LIF-treated regenerating muscles compared with control (no LIF) muscles (Fig. 3). Brief comments on the staining pattern for the different ECM antibodies follow.

Fibronectin. Strong interstitial staining for fibronectin was seen in the central necrotic core at all times in both LIF-treated (Fig. 3a) and control (Fig. 3b) muscles. Interstitial staining between relatively undamaged fibres at the edge of the crush lesion was also noted.

Tenascin-C. At the early stages of regeneration, little or no interstitial staining for tenascin-C was observed in the central necrotic core (not shown). Strong staining was noted at the edge of the crush injury and in areas of active myotube formation (Fig. 3c, d). Interstitial staining was weak around mature myotubes and also around myofibres in the undamaged regions.

Laminin. Very strong staining for laminin was seen in the basement membrane of undamaged muscle fibres and blood vessels (Fig. 3e, f). In the necrotic core of the crush lesion, staining was weaker and interrupted (not shown). As regeneration progressed, strong expression reappeared in capillaries in the central core and around developing myotubes.

Collagen type IV. Strong collagen type IV immunoreactivity was noted in undamaged muscle and blood vessels (not shown). At the early stages of regeneration, the staining in the necrotic core became patchy. Staining was strong around newly formed myotubes and blood vessels within the zone of regeneration (Fig. 3g, h).

Effect of culture substrate on BALB/c myoblast proliferation

The mitogenic effect of LIF (0.3 ng/ml) in vitro on the proliferation of primary BALB/c myoblasts grown on various ECM substrates (gelatin, fibronectin, collagen type IV and Matrigel) was quantified by measuring DNA replication by means of ^{3}H-thymidine incorporation (Fig. 4) and compared with control (no LIF) cultures.

Combined effects of LIF and ECM substrate

There was no effect of LIF on the rate of myoblast replication at any time in uncoated (Fig. 4a) wells and wells coated with gelatin, fibronectin or collagen type IV (data not shown). The addition of LIF to cultures grown on Matrigel (Fig. 4b) showed a highly significant increase in DNA replication ($P<0.01$) at 3 days, after which time, no differences were noted. The rate of cell replication of myoblasts grown in control (no LIF) media was significantly higher in uncoated wells compared with wells coated with Matrigel. This was not observed to any significant level for any other substrate used (data not shown).

Effect of LIF on BALB/c myoblast viability

The in vitro thymidine incorporation studies described above were conducted under sub-optimal conditions (5% v/v compared with 20% v/v FCS). To examine the contribution of cell death in the decline in incorporation levels after day 3, the effect of LIF on BALB/c myoblast viability in cultures was examined over the same 10-day period. Control (no LIF) cultures showed a gradual decline in the viability of cells over the 10 days (Fig. 5) dropping from 75% at day 1 to 32% at day 10. In the LIF-treated cultures, the decrease in myoblast viability was less significant, dropping from 89% on day 1 to only 54% by day 10. There was no statistical difference between the viability of cultures in control and LIF-treated cultures during the first 5 days but differences were noted at 7 and 10 days. At day 7, the viability of myoblasts

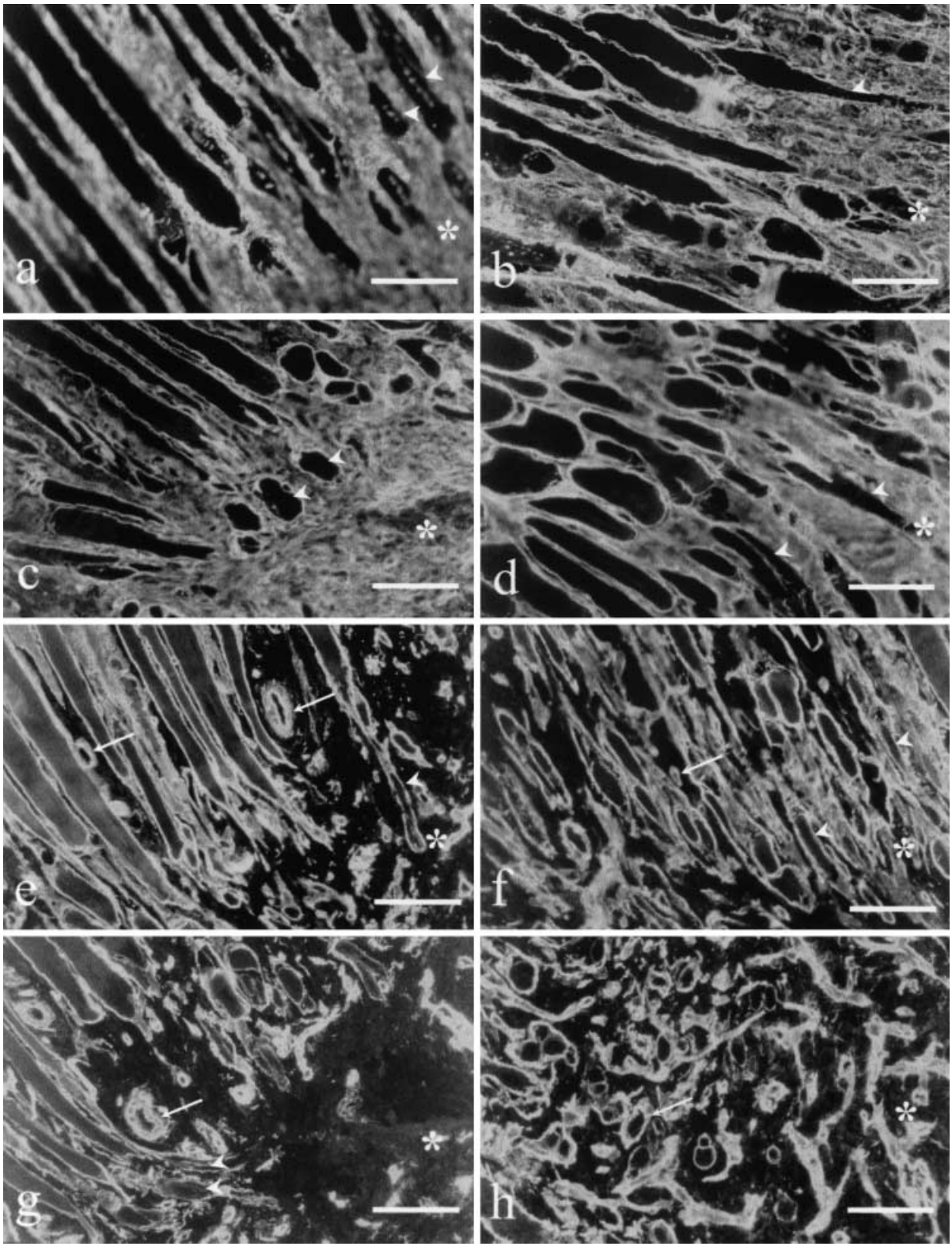
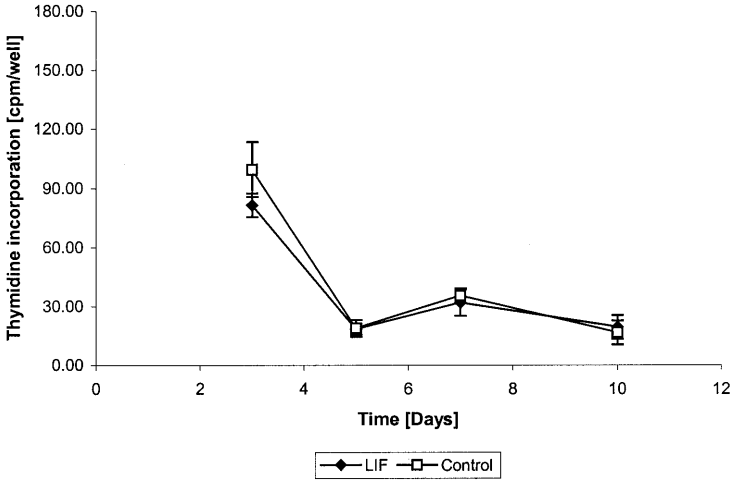
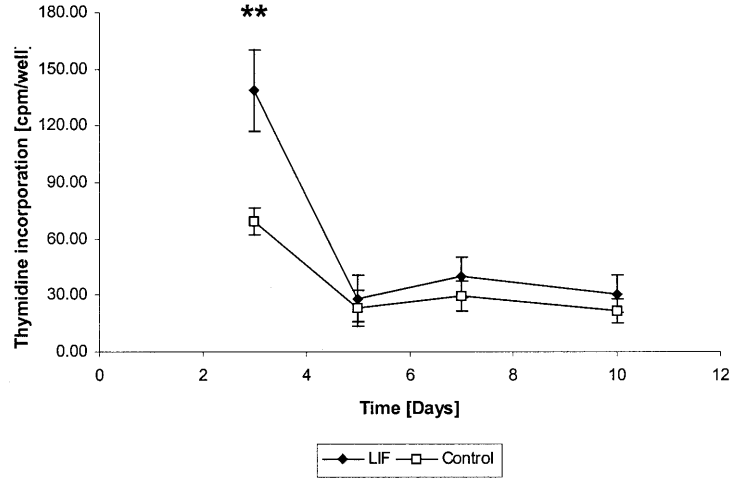


Fig. 3 Immunohistochemical staining of crush-injured BALB/c skeletal muscle at 5 days after surgery for LIF-treated (**a, c, e, g**) and control (no LIF) crushed muscles (**b, d, f, h**). Longitudinal frozen sections were stained with antibodies for fibronectin (**a, b**), tenascin-C (**c, d**), laminin (**e, f**) and collagen type IV (**g, h**). The central necrotic core (*asterisks*) of the crush lesion is indicated and, in many instances, surviving ends of damaged myofibres at the end of the crush lesion are present (*left*). Myotubes (*arrowheads*) and blood vessels (*arrows*) are also visible. *Bar* 100 µm



A



B

Fig. 4a, b The effect of LIF (0.3 ng/ml) on the proliferation of primary myoblast cultures. Thymidine incorporation was used to measure DNA replication at 12 h, 24 h, 2, 3, 5, 7 and 10 days after seeding primary BALB/c myoblast cultures grown on uncoated tissue culture plastic (**a**) or on Matrigel (**b**). All cultures were seeded in Hams F10 plus 5% (v/v) FCS. *Error bars* Standard deviation (n=4). Statistical differences between groups at a single time are shown (*P<0.05, **P<0.01)

in LIF-treated cultures was significantly higher, viz. 71% compared with 40% in control cultures (P<0.05). The viability on day 10 in the LIF-treated cultures was 54% (the same as at day 5) but this was still significantly higher (P<0.01) than the 33% viability seen in control (no LIF) cultures at day 10. Overall, in both LIF-treated and control (no LIF) cultures, there was a statistically significant decline in myoblast viability from days 1 to 5. However, in LIF-treated cultures, myoblast viability stabilised with no significant drop in myoblast viability between days 5 and 10. In control (no LIF) cultures, the viability of myoblasts continued to fall with a significant drop in viability from days 5 to 7 and from days 7 to 10 (Fig. 5).

Production of ECM components by myotubes

After 4 and 7 days in fusion media, myotubes were noted in all cultures grown on gelatin. No striking difference in the number and size of myotubes was observed between LIF-treated and control cultures at 4 or 7 days.

ELISA was used to quantify the expression of fibronectin, tenascin-C, laminin and collagen type IV, relative to the DNA content, by fusing BALB/c cultures in the presence and absence of LIF at 4 days (Fig. 6a) and 7 days (Fig. 6b) after the addition of fusion media. The standardisation with respect to DNA for the ELISA results took into account any variation in the number of nuclei between cultures (but did not indicate whether nuclei were present in myoblasts or myotubes). It should be noted that ELISA, as used here, only detects ECM molecules bound to the surface of cells or the culture dish and does not measure molecules in suspension. The cultures were observed closely and the analysis was performed on cultures that were not confluent and showed no evidence of cells piling up and allowing ready access for the antibody.

The production of fibronectin by LIF-treated cultures (Fig. 6a, b) was significantly higher than that by control

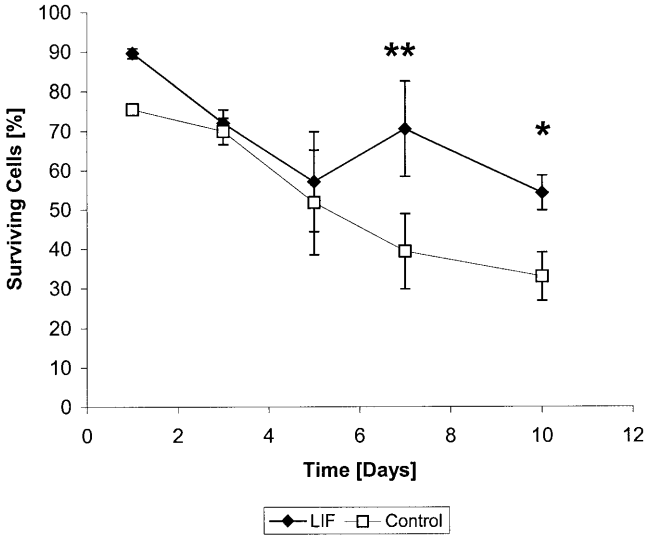


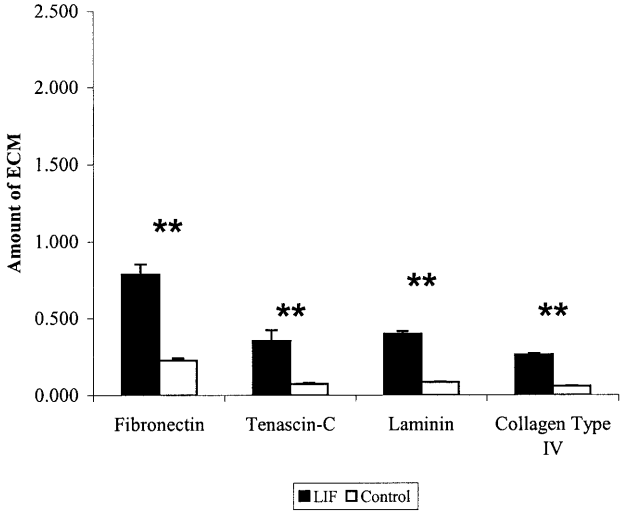
Fig. 5 The effect of LIF on myoblast viability was examined by using trypan blue exclusion. At time 0, equal number of myoblasts were seeded into 24-well plates and grown under sub-optimal conditions. At 1, 3, 5, 7 and 10 days in LIF-treated (0.3 ng/ml) and control (no LIF) cultures, the numbers of live cells were expressed as a percentage of the total number of cells in the culture (including free floating and attached cells). *Error bars* Standard deviation (n=4). Statistical differences between groups at a single time are shown (*P<0.05, **P<0.01)

cultures at day 4 (P<0.01) and day 7 (P<0.05). The production of tenascin-C, laminin and collagen type IV in LIF-treated cultures at day 4 (P<0.01) and day 7 (P<0.01) was also significantly increased.

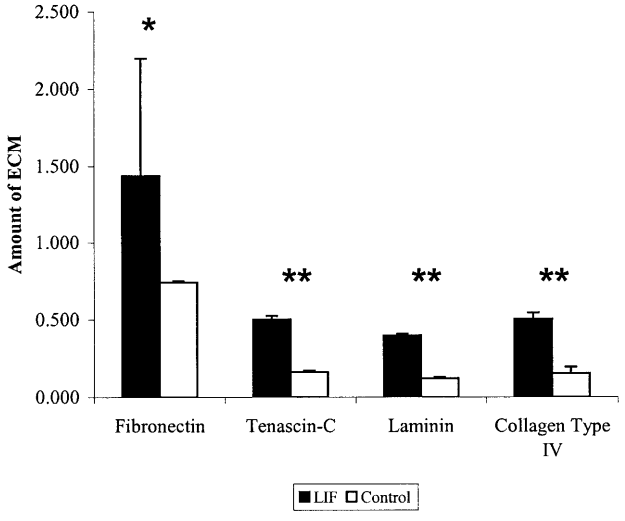
Discussion

Myoblast proliferation, ECM and LIF

Modulation of the ECM is a critical component of the regenerative response in skeletal muscle (Velleman 1998; Kherif et al. 1999). The present study addresses possible interactions between the ECM and LIF during myogenesis in vivo and in vitro. The in vitro data show no marked influence of various ECM substrates on myoblast proliferation in the absence of LIF, although some effects of ECM on the response to LIF have been seen (discussed later). We have not observed an increased rate of myoblast proliferation in LIF-treated cultures grown on uncoated tissue culture plastic, in apparent contrast to earlier reports (Austin and Burgess 1991; Austin et al. 1992). Whereas Austin and his colleagues reported an increased numbers of myoblasts (12-fold) at 4 days in LIF-treated mouse and human muscle cultures (grown on uncoated dishes), the numbers of myoblasts actually replicating at different time points were not quantitated. An increase in the number of myoblasts in a culture is a balance between previous myoblast replication/division and cell death. A greater number of myoblasts replicating at day 3 would result in more myoblasts at day 4 (assuming no cell death), even if replication subsequently decreases. Indeed, the present studies did show increased myoblast replication at day 3 (but not at day 5) in LIF-treated BALB/c myoblast cultures (compared with controls), grown on Matrigel. Whereas the numbers of replicating cells (myoblasts) were measured in the present study, the absolute numbers of myoblasts present at any time were not counted. LIF was also shown to maintain myoblast viability in the low serum conditions used here. Studies in other cell systems that show LIF signalling through gp130 and that STAT3 activates bcl-2 and decreases apo-



a – BALB/c at 4 days.



b – BALB/c at 7 days.

Fig. 6a, b ELISA quantitation (expressed as OD_{490nm}/µg DNA) of the amount of fibronectin, tenascin-C, laminin and collagen type IV bound in fusing BALB/c primary cultures. The effect of LIF (0.3 ng/ml) was compared with control (no LIF) cultures at 4 or 7 days after addition of fusion media. **a** BALB/c cultures at 4 days. **b** BALB/c cultures at 7 days. Statistical differences between groups at a single time are shown (*P<0.05, **P<0.01)

ptotic cell death (Fukada et al. 1996) might help to explain our in vitro "rescue" of myoblasts by LIF, although no direct measure of apoptosis has been undertaken. It is tempting to speculate that LIF affects myoblast cultures (largely or in part) as a survival (anti-apoptotic) factor. Therefore, it is possible that the transient increase in DNA replication (at 3 days) combined with enhanced viability (or a lower level of cell death) in the presence of LIF, as noted here, could result in an increased absolute number of myoblasts present, as previously reported (Austin and Burgess 1991; Austin et al. 1992). Such different approaches to analysis (combined with variations in the types of myoblasts used) may well account for the apparent discrepancy in results from studies relating to the timing of the proliferative response of myoblasts to LIF. A combined survival (and mitogenic) effect mediated by LIF associated with increased myoblast replication induced by bFGF might also account for additive effect of these two factors on myoblast number as reported previously (Austin et al. 1992).

The kinetics of myoblast proliferation in response to LIF in vitro appear to differ from other mitogenic growth factors such as bFGF, as the initial proliferative response to LIF is delayed by 2–3 days and is sustained (Austin and Burgess 1991; Austin et al. 1992) compared with the rapid (within 48 h) and transient increase in proliferation seen with bFGF (Gospodarowicz et al. 1976; Allen et al. 1984). Previous autoradiographic studies show that the onset of myoblast replication normally occurs approximately 30 h after crush injury of BALB/c muscle and replication increases between 48 h to 72 h after which time replication declines (Grounds and McGeachie 1989). This is consistent with the pattern of myoblast replication seen in regenerating LIF-treated and control muscles in the present study (retrospectively derived from the autoradiographic analysis of labelled myotube nuclei). The increased myoblast proliferation seen at 5 days but not 3 days in LIF-treated muscles suggests that, after the initial activation and proliferation of satellite cells, LIF transiently stimulates myoblast replication but this effect is short lived as differences are not apparent between test and control muscles at 7 days. These in vivo data of a "delay" in LIF stimulation of myoblast proliferation resemble the in vitro observations in our study.

Despite the extended proliferation noted in LIF-treated muscles, in vivo histological evaluation of myotube formation indicates no delay in the fusion of myoblasts to form myotubes in vivo. This contrasts with animal models such as the MyoD(–/–) mouse where a sustained level of myoblast proliferation results in a very marked 2- to 3-day delay in myotube formation (White et al. 2000). It is interesting to note that although myoblast numbers are significantly increased in MyoD(–/–) muscle, this does not result in enhanced new muscle formation (Megeney et al. 1996; White et al. 2000). Two other studies in which myoblast replication is increased by hepatocyte growth factor (Miller et al. 2000) or ultrasound (Rantanen et al. 1999) also report that increasing the number of myoblasts does not necessarily result in improved regeneration, although studies by DeRosimo et al. (2000) in which the addition of exogenous myoblasts does enhance overall regeneration provide support for such an approach.

Myotube formation and LIF in vitro and in vivo

No effect of LIF on myotube formation has been observed with respect to myotube size or timing of myotube formation, either in vitro or in vivo, in contrast to earlier reports (Vakakis et al. 1995). Previous in vitro studies showing a beneficial effect of LIF on myotube formation have involved the use of uncoated tissue culture plastic and C57BL10/ScSn myoblasts (Vakakis et al. 1995), whereas we have employed gelatin (denatured collagen-1) and myoblasts derived from BALB/c mice. Thus, the differences might be attributable to the culture substrate and the strain of myoblasts used. Although a number of significant differences have been seen in the amount of ECM produced in the presence of LIF (see below), these differences do not appear to affect myoblast fusion significantly in vitro. The most striking differences in ECM production in response to LIF have been noted at 4 days, a time when mainly myoblasts rather than myotubes are present in the cultures. Since LIF receptors are only expressed on myoblasts but not myotubes or fibroblasts (Bower et al. 1995; Kami et al. 2000), it seems unlikely that LIF directly affects myotube growth or maturation.

Morphometric analysis has not revealed any effect of LIF on the size of myotubes formed in regenerating skeletal muscle of BALB/c mice. This observation is in direct contrast to earlier studies in which LIF treatment after crush injury increases the size of regenerated myofibres (Barnard et al. 1994; Kurek et al. 1997). However, in support of our observations, recent studies in rat skeletal muscle regenerating after bupivacaine injection report that LIF has no effect on regenerating myofibre size or functional capacity (Gregorevic et al. 2000a, 2000b). There are two main technical differences between these reports that may contribute to the contradictory observations. Barnard et al. (1994) and Kurek et al. (1997) used C57Bl/10ScSn and *mdx* mice, whereas we investigated BALB/c mice and Gregorevic et al. (2000a, 2000b) studied rats. It is well documented that the regenerative capacity of BALB/c mice after crush injury is less effective than for SJL/J mice (Mitchell et al. 1992) and C57Bl muscle shows an intermediate capacity (unpublished data). The lack of any effect of LIF on myotube size in this study might reflect a lack of sensitivity of BALB/c skeletal muscle to LIF, although other data indicate similar responses of myoblasts from various mouse strains to growth factors. The second technical difference is that different delivery systems for LIF were employed, although it seems unlikely that this is crucial. In our study, LIF was released from alginate gels, whereas the other studies used osmotic pumps (Barnard et al. 1994; Kurek

et al. 1997) or systemic intra-peritoneal administration (Gregorevic et al. 2000a, 2000b). Alginate gels lie in intimate contact with the damaged muscle, whereas osmotic pumps deliver LIF directly into the crush injury via a cannula; both of these approaches rely on the local diffusion of LIF into the muscle compared with intra-peritoneal injection, which relies on delivery via the circulation. All three techniques have been effective in previous studies (Barnard et al. 1994; Ikeda et al. 1996; Kurek et al. 1996a, 1996b, 1996c, 1997; Gregorevic et al. 2000a, 2000b) and LIF released from alginate rods clearly increases myoblast proliferation at 5 days (discussed above) in the present study. Furthermore, related investigations on dystrophic muscle show no effect of LIF on myotube size in diaphragms of dystrophic *mdx* or control C57BL10/ScSn mice after 3 months (J. D. White et al., in preparation). Overall, these data do not support a consistent and major role for LIF in enhancing myotube formation in vivo.

LIF and expression of ECM in vivo and in vitro

Extensive remodeling of the ECM, detectable by immunohistochemistry, is found during tissue repair and inflammation including inflammatory bowel disease (MacDonald et al. 1990; Riedl et al. 1992) and wound healing (Raghow 1994) during crush injury of skeletal muscle (Grounds et al. 1998). The changes in ECM expression during skeletal muscle regeneration have been well described elsewhere and our observations correspond closely with these published in vivo data (Grounds et al. 1998; Sorokin et al. 2000). Overall, no affect of LIF on the pattern of ECM expression in vivo has been noted in the present study.

In both LIF-treated and control (no LIF) crush-injured muscles, fibronectin and tenascin-C are highly up-regulated in necrotic and regenerating areas and, as observed in other tissues (Kaarteenaho-Wiik et al. 2000), tenascin-C is prominent in areas of newly formed fibrosis. The interstitial ECM components, fibronectin and tenascin-C, are up-regulated in inflammation and often co-localise in tissues (Chiquet-Ehrismann et al. 1988; Chiquet-Ehrismann 1990) and abundant tenascin-C expression in adult tissues has been noted under conditions that are associated with high rates of cell turnover and migration (Haapasalmi et al. 1996) of incoming fibroblasts, endothelial and myogenic cells. Increased tenascin-C has also been reported in the vicinity of new myotubes (Mackie et al. 1988; Daniloff et al. 1989; Irintchev et al. 1993; Grounds et al. 1998). The pattern of expression of tenascin-C in several muscular dystrophies correlates with t he presence of macrophages and muscle regeneration (Settles et al. 1996) and it is of interest that in situ hybridisation does not reveal any production of tenascin-C by muscle cells, indicating that non-muscle cells synthesise the majority of tenascin (Gullberg et al. 1998). This suggests that, in our primary muscle cultures, fibroblasts must be largely responsible for the production of tenascin-C. The proportion of myoblasts in such cultures (as calculated by desmin staining) is about 50% (data not shown), a level that would closely resemble the in vivo situation and that corresponds well to other studies (Maley et al. 1995). In tissue culture, a number of differences in the production of ECM have been noted. A marked difference in the amount of fibronectin between days 4 and 7 has been seen, the lower levels of fibronectin at day 4 being in keeping with observations that fibronectin is expressed by myoblasts and myotubes but is down regulated at the time of myotube formation (Gardner and Fambrough 1983).

The effect of LIF on skeletal muscle regeneration

Histological studies by Kurek et al. (1996a, 1997) have shown that LIF (delivered via osmotic pump and canula directly into the injured muscle) results in a 50% decrease in the area occupied by connective tissue and mononuclear cells 5 days after crush injury. Our observations after crush injury, as demonstrated by immunohistochemical staining of the ECM components fibronectin and tenascin-C, do not reflect any increased interstitial component. The expression of collagen type I, a major interstitial component, has not been quantitated in the present study; however hydroxyproline quantitation of the collagen content of LIF-treated *mdx* diaphragms have shown no difference between LIF and control *mdx* diaphragms (Austin et al. 2000). This recent quantitative study combined with our immunohistochemical analysis of fibronectin, tenascin-C, laminin and collagen type IV (where there is no difference between LIF-treated and control muscles) does not support a significant role for LIF in the modification of ECM in skeletal muscle regenerating in vivo.

These detailed studies with LIF have specifically examined the influence and production of ECM molecules, myoblast proliferation in vitro and in vivo and myotube formation. Whereas some differences in ECM production by myotubes in vitro have been noted in response to LIF, the production of ECM in vivo after crush injury is not significantly altered. These combined data do not support a consistent beneficial role for LIF on myotube formation. Some subtle effects of the ECM substrate have been noted on myoblast proliferation in response to LIF. The consistent finding is that LIF maintains myoblast proliferation. This mitogenic effect combined with enhanced myoblast viability probably accounts for the increased myoblast numbers reported in the presence of LIF.

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