

Leukaemia Inhibitory Factor and Other Cytokines as Factors Influencing Regeneration of Skeletal Muscle

John B. Kurek, John J. Bower⁽¹⁾, Jason D. White⁽¹⁾, Catriona M. Muldoon⁽¹⁾ and Lawrence Austin⁽¹⁾

Amrad Pharmaceutical Research and Development, Richmond, Victoria, and (1) Melbourne Neuromuscular Research Centre, St. Vincent's Hospital, Fitzroy, Victoria, Australia

Abstract

When skeletal muscle is injured, mononuclear muscle precursors, the satellite cells, are activated and begin to proliferate and then fuse with each other to form new multinucleated myotubes, which eventually become the mature muscle fibres. The process of muscle regeneration is regulated by locally produced growth factors that control cellular proliferation and differentiation. Several polypeptide growth factors have been implicated as regulators of myogenic cell proliferation and differentiation during development and after injury. Leukaemia inhibitory factor (LIF), interleukin-6 (IL-6) transforming growth factor- α (TGF- α), basic fibroblast growth factor (bFGF), and insulin like growth factor-1 (IGF-1), are five cytokines which strongly stimulate the proliferation of muscle myoblasts *in vitro*, with LIF being the most effective. These actions suggest that they have a regulatory role in the development and regeneration of muscle.

The muscle crush model in mice was used to test the effects of these factors in muscle regeneration. Growth factors were administered to injured muscle using osmotic pumps implanted subcutaneously and the growth factor was continuously delivered to the site of injury via a cannula. The results show that LIF greatly increased the rate of muscle regeneration in wild type and LIF knockout mice. LIF stimulated the formation of larger muscle fibres, while not stimulating proliferation of resident fibroblasts. A similar effect was seen when muscle of the *mdx* mouse was treated with LIF. In contrast, no stimulation of regeneration was seen with IL-6, TGF- α or bFGF. This myotrophic action indicates that LIF contributes to muscle regeneration, and is the only factor to have a positive effect when applied exogenously after injury. Together with its known neurotrophic action, LIF is a potential therapeutic agent for the treatment of neuromuscular disease.

In summary, this review examines the role of growth factors in muscle after injury and demonstrates a significant role for LIF in the regeneration process.

Key words: leukaemia inhibitory factor, interleukin-6, transforming growth factor- α , insulin like growth factor, fibroblast growth factor, myoblast, muscle regeneration.

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The Role of Growth Factors in Myoblast Proliferation and Differentiation

Growth factors are defined as secreted regulatory proteins that control the survival, growth, differentiation and effector function of tissue cells. Most growth factors are small proteins of fewer than 200 amino acids and are active at picomolar concentrations. Growth factor signals are mediated by specific cell surface receptors; therefore, an effective response requires, at minimum, the presence of both the growth factor and its

receptor. Much research has centred on the identification of factors, which are released by injured muscle. Extracts from crushed muscles stimulate the proliferation of satellite cells *in vitro* where extracts from intact muscle, lung and liver do not [11]. A number of growth factors have been shown to stimulate satellite cell proliferation *in vitro*. These include basic fibroblast growth factor (bFGF), leukaemia inhibitory factor (LIF), insulin like growth factors I and II (IGF I and II) and interleukin 6 (IL-6) transforming growth factor- α (TGF- α) and platelet derived growth factor

(PDGF) [6,7]. Factors such as transforming growth factor- β (TGF- β) have a negative effect on proliferation and inhibit the fusion of myoblasts to myotubes. Many of these factors are produced in response to injury and in situations of myopathy.

There are clear differences between the effects of these factors *in vitro*. One major factor relevant to *in vivo* effects is that bFGF, TGF- α and IL-6 also stimulate fibroblast growth while LIF has no effect on fibroblasts. In addition, bFGF, TGF- α and IL-6 all suppress fusion of myoblasts to myotubes while LIF induces rather larger myotubes and IGF1 stimulates the fusion process. The actions of LIF and bFGF are totally additive, suggesting very different intracellular mechanisms are involved. It is likely that there are multiple regulatory mechanisms affecting satellite cell proliferation and differentiation *in vivo*, and these factors have both positive and negative influences *in vitro*. Regulation of the recruitment or activation of satellite cells into the cell cycle is not well understood, particularly *in vivo*. Once satellite cells are activated, the inhibition and stimulation of proliferation and differentiation appears to be controlled by multiple factors.

Satellite cells in adult muscle are quiescent, or in the G₀ phase of the cell cycle [12], and are thought to be acted on by at least two different types of growth factor before commencing sustained proliferation. The cell must first become induced to enter the cell cycle and undergo mitosis by a competence factor, and then stimulated to traverse the remainder of the cell cycle and undergo mitosis by a progression factor. Other growth factors would play a role in maintaining the quiescence of satellite cells in mature muscles in G₀ and suppressing further divisions after regeneration is complete. Historically, the most extensively studied agents influencing myoblast proliferation and differentiation are the fibroblast growth factors (FGFs), the insulin like growth factors I and II (IGFs) and the transforming growth factor- β (TGF- β) family.

The mechanism of activation of the cells and the factors that guide their behaviour are not clearly understood. The availability of polypeptide growth factors produced either by the muscle precursor cells, or by associated non-muscle cells, may be involved in modulating the behaviour of satellite cells and myoblasts. Work by Bischoff [13], has shown that satellite cells in contact with the plasmalemma of a viable myofibre will proliferate when exposed to an extract from crushed muscle, but although fusion competent, they do not fuse with the myofibre. This indicates that the myofibre directly influences the behaviour of the satellite cells attached to it.

Fibroblast Growth Factors (FGF)

There are many forms of FGF, but the most studied are basic FGF and acidic FGF. Both are single polypeptide chains of 146 and 140 amino acids respectively. Basic FGF is the most well known competence factor, is more widely distributed and is the more potent form [30]. Basic FGF stimulates the proliferation of cultured satellite cells from a variety of species and inhibits their differentiation [1, 2, 7, 20, 33, 54]. Basic FGF mRNA has been detected in myoblasts just prior to fusion and in myotubes of primary muscle cell cultures [36]. Receptors for basic FGF have been detected in myoblasts, but not in myotubes [60]. Although little is known about the physiological role of FGF in muscle, it is present *in vivo*, and its level appears to vary with the physiological changes in the muscle. Basic FGF has been found in the basal lamina of normal adult mouse muscle and the amount is elevated in *mdx* mouse muscle, in which there is increased regeneration and satellite cell proliferation [3, 20]. Direct muscle injury results in elevated levels of bFGF mRNA in both degenerating and regenerating myotubes [4, 36].

Insulin-Like Growth Factors (IGF-I + II)

The IGFs are a family of small single chain peptides, similar in structure to insulin [31]. IGF-I (70 amino acids) is a mediator of growth hormone action, while IGF-II (67 amino acids) may function to stimulate cell proliferation during development. The IGFs are progression factors. Quiescent satellite cells on single muscle fibres *in vitro* do not proliferate in response to IGFs but once activated by FGF will respond to IGFs [12, 13]. IGF-I stimulates the proliferation of cultured satellite cells and promotes their differentiation, with IGF-I being more potent than IGF-II [1, 23, 24, 29]. The action of the IGFs is mediated by cell surface receptors. Receptors have been detected on satellite cells, myoblasts and myotubes *in vitro* [22, 25, 64]. Skeletal muscle is also a producer of IGFs. Myoblasts express mRNA for IGF-I and II, and when they differentiate into myotubes, IGF-II mRNA levels increase [19, 26, 40, 42, 68]. There is also local production of IGF-I and II mRNA in regenerating skeletal muscle following injury [26, 40, 52], following denervation [18] and during reinnervation [32]. If injured muscle is treated with IGF-I antibodies, regeneration is inhibited [50].

Transforming Growth Factor- α (TGF- α)

One of the better characterised mammalian growth factors is Transforming Growth Factor- α (TGF- α). TGF- α , a secreted polypeptide of 50 amino acids, is a member of a small family of structurally related growth factors that includes epidermal growth factor (EGF), amphiregulin, heparin-binding EGF (HB-EGF), and betacellulin [65]. In cell culture systems, TGF- α

Cytokines influencing regeneration of skeletal muscle

stimulates the proliferation of a range of cell types, including many epithelial and mesenchymal cells, with the notable exception of haemopoietic cells. All the biological properties of TGF- α are elicited through the EGF receptor. TGF- α synthesis has been reported in activated macrophages [61]. The abundant presence of macrophages at the sites of inflammation and wound healing and the reported effects of TGF- α on myoblast proliferation [6, 7] strongly suggest a role for TGF- α in muscle regeneration. In addition, TGF- α is able to induce proliferation of other cell types that have EGF/TGF- α receptors, such as fibroblasts, which are intimately associated with muscle cells. EGF has no effect on myoblasts *in vitro*, but it competes with TGF- α for receptors on myoblasts [16].

Transforming Growth Factor-Beta (TGF- β)

Recent studies have identified several factors as inhibitors of satellite cell proliferation. The best studied is Transforming growth factor-beta (TGF- β), a homodimer of two 112 amino acid chains, first purified from platelets [30]. TGF- β is an inhibitor of myogenic proliferation and differentiation [1, 28, 43, 55, 59]. It blocks all measured aspects of myogenic differentiation; fusion, elevated creatine kinase activity, appearance of acetylcholine receptors, transition from b- to a-actin, and the expression of other muscle specific genes. The neutralising of TGF- β in injured muscle with antibodies led to an increased number of small regenerating fibres [50], indicating a direct *in vivo* role for this molecule.

Platelet Derived Growth Factor (PDGF)

Platelet derived growth factor (PDGF) stimulates proliferation of many mesodermal cells and is implicated in wound healing [63]. PDGF stimulates proliferation of myoblasts from embryos and myogenic cell lines [73]. It is also a chemoattractant for embryonic myoblasts [70]. Growth of mouse satellite cells *in vitro* are also weakly stimulated by PDGF [6].

Interleukin-6 (IL-6)

IL-6 was first recognised as a T-cell derived factor acting on B cells to induce immunoglobulin secretion [38, 41]. Since then IL-6 has been found to be a pleiotropic cytokine, produced by many different cell types. The human form is made up of 184 amino acids. Interleukin-6 (IL-6) is a cytokine with pleiotropic activities that plays a central role in host defense. IL-6 can exert growth-inducing, growth-inhibitory, and differentiation-inducing activities, depending on the target cells. Our knowledge of the action and role of IL-6 in muscle is rather limited. IL-6 mRNA is constitutively expressed at low levels in normal muscle [15] and it is up-regulated in response to injury and in muscle of the *mdx* mouse [48]. While it is unclear whether this low-level expression is of biological significance, it is possible that it reflects a local mode of

action of IL-6. It has been shown that IL-6 stimulates muscle precursor cell proliferation *in vitro* [6, 7], and when added together with LIF, the effect is not additive, but only slightly greater than that of IL-6 alone.

LIF as a Regeneration Factor in Muscle

Leukaemia inhibitory factor (LIF) is a secreted polyfunctional cytokine of 180 amino acids, that elicits a diversity of biological effects on many cell types. Rather than exerting a single biological effect, LIF is pleiotropic in its action, and has a broad range of effects on many cell types, including haemopoietic cells, embryonic stem cells, primitive germ cells, hepatocytes, neurons, adipocytes, myoblasts and osteoblasts. [71]. In muscle LIF has been shown to markedly stimulate the proliferation of both mouse and human muscle precursor cells (myoblasts) in culture, while not stimulating resident muscle fibroblasts [6, 7]. The response to LIF is greater than that observed with other growth factors, including bFGF, IGF-1, PDGF, IL-6 and TGF- α . When present together with bFGF or TGF- α , however, the effects are totally additive, with proliferation exceeding that normally seen with either factor alone. The response to LIF is long term, with the proliferative effect not seen until 4-6 days after addition of the factor. There are specific receptors for LIF on myoblasts (KD ~400 pM). The number of receptors decreases as the *in vitro* cell density increases, and once the myoblasts fuse and become myotubes, LIF receptors are no longer expressed, and the myotubes do not respond to LIF [16]. When myoblasts are fused in the presence of LIF *in vitro*, LIF does not suppress fusion [69]. Normal myotubes were formed as determined by normal patterns of expression for acetylcholine receptors (AChRs), and myosin light chains. The actions of LIF in muscle appear to be to stimulate muscle precursor cell proliferation, while not influencing terminal differentiation. LIF mRNA is expressed in various tissues during embryonic development [58], but its precise role in the development of muscle is not known. LIF related ligands, however, may be involved in the developmental process. The receptor subunit gp130 is a ubiquitously expressed signal-transducing receptor component shared by LIF, oncostatin M, ciliary neurotrophic factor, cardiotrophin-1, IL-6 and IL-11. Recent reports of the targeted disruption of gp130 implicate a function for this molecule in diverse systems [74]. The key role of the ligand specific β subunit (LIFR β) has also been demonstrated in recent studies with the LIFR β knockout mouse, which is also a lethal defect. [53]. In skeletal muscle, Bower *et al* [53] report the presence of a high affinity LIF receptor on myoblasts. These receptors appear to be under negative feedback control as time- and concentration-dependent down-regulation of these receptors occurs in cell culture in the continued

presence of LIF. There is up-regulation of LIFRb following denervation *in vivo* [39]. In wild-type mice, the timecourse of LIF mRNA expression, after muscle injury, correlates with the period of tissue repair. After muscle injury, LIF mRNA is expressed in mononucleated cells and damaged muscle cells, as well as in the Schwann cell-like cells of intramuscular nerves [45, 48]. More recent work from our laboratory indicates that there is endogenous expression of LIFRb in skeletal muscle after injury (unpublished).

LIF Treatment Stimulates Muscle Regeneration

Gene targeting of the LIF gene has demonstrated that LIF is not a limiting factor in muscle development, since LIF knockout mice develop normally and the primary deficit is in the failure of the blastocyst to implant [66]. Initial experiments examined the regenerative capacity of crush injured muscle of the LIF knockout mouse. This was compared with the regeneration that occurs in wild type mice. To this end, the *vastus lateralis* muscle of both LIF knockout and wild type mice were subjected to the same crush injury. After 7 days the crushed muscle was removed, fixed and transverse sections cut and stained. The diameter of the regenerating myotubes was measured and the results between the two groups compared [47]. Figures 1A & 1B show the muscle histology 7 days after a crush injury in wild type and LIF mice. Muscle sections from wild type and knockout animals look similar. There are areas of necrosis, infiltration of non-muscle cells and there are numerous regenerating myotubes, identified by their centralised nuclei. Immunohistochemistry was performed on sections of wild type muscle 7 days after a crush injury. They were stained for desmin, a muscle specific cytoskeletal protein. Only the regenerating muscle fibres were stained. The necrotic fibres and non-muscle components were not stained. Muscle sections from the knockout mouse showed similar staining patterns (results not shown). The muscle crush model was used to test the effect of LIF on muscle regeneration. Growth factor was administered to injured muscle using osmotic pumps implanted subcutaneously and the growth factor was continuously delivered to the site of injury at the desired concentration for 7 days via a catheter as described by [9]. In the present study, this was extended to include testing the effects of LIF on the muscle of LIF negative mutants. Image analysis (Table 1), shows that there was a significant difference in the average fibre diameter between the two groups. The regenerating fibres in the LIF knockout mouse were 25% smaller than the corresponding fibres in the wild type mouse ($p < 0.05$). Table 2 shows that there was no difference in the fibre density between the two groups [47].

Figure 1C & 1D shows the histology of the regenerating *vastus lateralis* muscle in wild type and LIF $-/-$ mice after 7 day treatment with LIF. There were a larger number of large multinucleated regenerating myofibres in the perfused area of both wild type and LIF $-/-$ muscle compared with the 7 day control groups. Image analysis (Table 1) shows that after 7 days the average fibre diameter increased by 53% in the wild type and 93% in the knockout, over the control groups ($p < 0.01$). There was also a corresponding increase in the volume density in the LIF treated groups (Fig 2). The area occupied by regenerating fibres increased after LIF treatment in both wild type and mutant groups. The wild type volume density increased from 43 to 54% ($p < 0.05$) of the total regenerating area, and the volume density in the LIF $-/-$ from 38 to 64% ($p < 0.01$) after LIF treatment. In the knockout, the area occupied by mononuclear cells and connective tissue was half that of the area occupied by the muscle fibres. Interestingly the density of fibres was similar in both wild type and LIF $-/-$ mice after LIF treatment (Table 2), therefore the increase in volume density was attributed to an increase in fibre diameter and not to an increase in fibre number.

Immunohistochemistry was performed on sections of muscle from wild type and knockout mice after LIF treatment. Figure 3 shows a desmin-stained section from each group. All the regenerating muscle fibres were stained for desmin, and the pattern of staining indicated that the non-muscle component was reduced in both cases. This is in contrast to the desmin staining seen in sections of the wild type control group, where there was a greater non-muscle component (results not shown).

Table 1. Determination of average fibre diameter of wild type and LIF knockout muscle after PBS, LIF, IL-6 and TGF- α treatment. The numbers are the mean diameter $\pm$ SD of at least 2500 fibers. $n = 4$ mice in each group. Statistical comparison between growth factor and PBS treated groups was assessed using a two-tailed Student *t*-test ($p < 0.05$) and single factor analysis of variance.

Muscle type	Average fibre diameter (mm)			
	Injured + PBS	Injured + LIF	Injured + IL-6	Injured + TGF- α
Wild type	42 $\pm$ 5	64 $\pm$ 7	47 $\pm$ 2	45 $\pm$ 6
LIF Knockout	32 $\pm$ 4	62 $\pm$ 5	-	-
Statistical analysis				
LIF $-/-$ + PBS	vs	LIF $-/-$ + LIF	$p < 0.01$	
LIF $-/-$ + PBS	vs	WT + PBS	$p < 0.05$	
WT + PBS	vs	WT + LIF	$p < 0.01$	
WT + PBS	vs	WT + IL-6	$p < 0.05$	
WT + PBS	vs	WT + TGF- α	$p < 0.05$	

Cytokines influencing regeneration of skeletal muscle

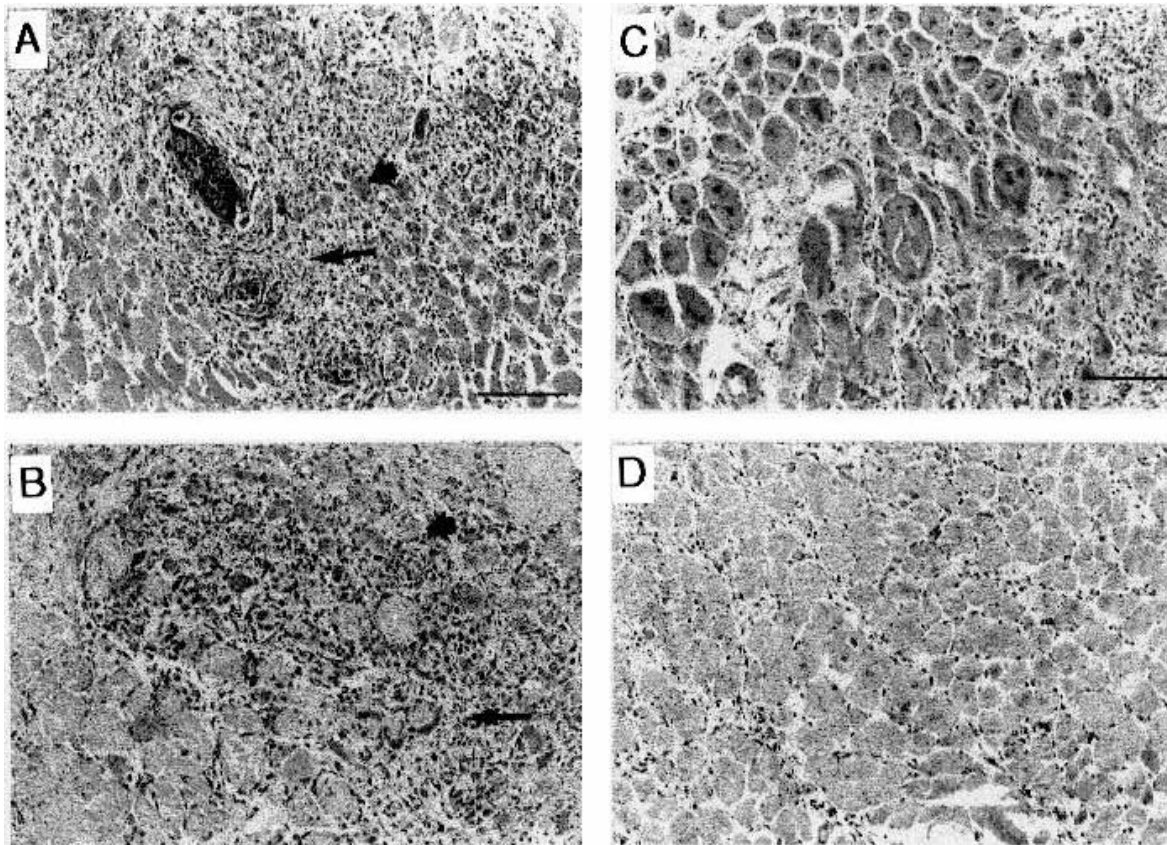


Figure 1. Cross section (x200) of wild type and LIF $-/-$ vastus lateralis muscle 7 days after a crush injury, before and after LIF treatment. Sections stained with haematoxylin and eosin. Top left (A) Wild type; bottom left (B) LIF knockout. Wide arrow indicates regenerating fibres; narrow arrow indicates mononuclear cells and connective tissue. Top right (C) Wild type and LIF; bottom right (D) LIF knockout and LIF; bar: 100 μ m.

Since LIF was the only factor to have a positive effect on muscle regeneration in wild type and LIF knockout mice, it was of interest to test the effects of LIF in diseased muscle. The final group of *in vivo* experiments were therefore concerned with muscle regeneration in the *mdx* mouse, which is the most common animal model of human Duchenne muscular dystrophy (DMD). It shares the same genetic defect and suffers the same vigorous muscle necrosis, but unlike the muscles in human DMD, the skeletal muscle fibres of the *mdx* mouse undergo regeneration enabling them to remain active and survive. LIF was infused into the *vastus lateralis* muscle of the *mdx* mouse continuously for 7 days [49]. The muscle was, however, not subjected to a mechanical trauma. The objective was to observe any changes to the continuous endogenous muscle regeneration that is occurring as a result of the pathology of the disease.

Figure 4 shows the histology of the *vastus lateralis* muscle of the *mdx* mouse before and after treatment with LIF. The control group (Fig 4A) shows small groups of regenerating fibres with central nuclei surrounded by normal muscle fibres of various diameters containing both peripheral and centralised nuclei. After 7 days continuous LIF treatment, the

histology shows numerous multinucleated regenerating muscle fibres in the perfused area of the muscle. There were also small mononucleated cells and areas of connective tissue (Fig 4B). Image analysis (Table 3), shows that after 7 days the average fibre diameter increased by 22% over the control groups ($p < 0.05$). Table 4 shows that the LIF delivered during the first 7 days still had an effect after 14 days, with the average fibre diameter 30% greater than the PBS treated groups at both time points ($p < 0.01$). Since LIF stimulates the formation of larger myotubes, the density of regenerating muscle fibres in the PBS and LIF treated groups was determined. Table 4 shows that after 7 days LIF treatment the number of regenerating fibres in the perfused area had not changed, but after 14 days it had increased two fold. This was a surprising observation since in wild type animals, LIF treatment of injured muscle only results in an increased average fibre diameter after 7 days. It should be noted that the wild type was not studied after 14 days.

The histological features after LIF treatment also include increased numbers of mononucleated cells and an increased extracellular component. Immunohistochemistry was used to assess whether any of these mononuclear cells were myogenic in origin. Fig 4C

Cytokines influencing regeneration of skeletal muscle

Table 2. Density of regenerating fibres in wild type and LIF knockout muscle after treatment with PBS, LIF, IL-6 and TGF- α . There was no significant increase in the number of regenerating fibres after LIF treatment when compared to the control groups. (* $P > 0.05$). There was a significant decrease in the number of regenerating fibres after treatment with IL-6 and TGF- α (# $P < 0.01$).

Muscle type	No. regenerating fibres $\pm$ SD / 100 mm ² area			
	Injured + PBS	Injured + LIF	Injured + IL-6	Injured + TGF- α
Wild type	1.6 $\pm$ 0.1	1.5 $\pm$ 0.02*	1.0 $\pm$ 0.12#	0.94 $\pm$ 0.06#
LIF Knockout	1.7 $\pm$ 0.2	1.5 $\pm$ 0.12*	-	-

& 4D shows LIF treated *mdx* muscle stained positive for desmin, a muscle specific cytoskeletal protein. A significant number of the mononuclear cells had stained, indicating they were muscle precursor cells. After 14 days most of the available myoblasts have proliferated and fused, leaving larger fibres. There were, however, some mononuclear cells that did not stain, indicating that they were non-muscle cells. Some of the mononuclear cells that did not stain could be polymorphonuclear leukocytes and macrophages, since insertion of the cannula causes a small focal injury to the muscle, which would result in some localised necrosis. This was not so evident in the PBS treated groups, and was unexpected. It may be a result of LIF stimulating the infiltration of these

cells to enter the damaged muscle.

The most important observation of this study was that there was less endogenous regeneration in muscle from LIF $-/-$ mice than in the corresponding wild type animal. This directly implicates LIF in muscle regeneration. Treating the LIF deficient muscle with LIF stimulates regeneration to produce muscle fibres that are the same size as those of the wild type group treated with LIF, whereas they are significantly smaller in the absence of LIF. This shows that the only thing missing in the LIF knockout animal is LIF. That is, there have been no irreversible changes in these animals, which hinder their regeneration, and addition of LIF results in wild type regeneration rates. This also suggests that LIF acts as a trauma factor for muscle. If it did have a major role in muscle development then perhaps other differences would show up, such as smaller muscle mass or an inherently decreased capacity to regenerate. This is not to say it has no role during development, but other ligands may be acting in its place to compensate. One of the most striking features in the muscle of LIF $-/-$ mice that have been treated with LIF is the change in volume density. The regenerating muscle fibres take up a higher percentage of the regenerating area than that observed in the other groups. Interestingly, the number of regenerating fibres does not change after LIF treatment, and LIF only seems to be involved in the regulation of muscle fibre size. This presumably occurs through increased myoblast production leading to fusion to existing as well as new fibres. LIF may be acting directly on the pool of satellite cells that are activated when an area of injured muscle undergoes necrosis and begins to regenerate [10, 12]. Support for this proposal comes from the finding that LIF stimulates myoblast proliferation *in vitro* and that myoblasts express LIF receptors *in vitro* [16]. Myotubes do not express LIF receptors suggesting that LIF has no action on intact muscle. The LIF stimulated growth of myoblasts in culture could be analogous to the situation in muscle following trauma.

The results show that LIF increases the rate of regeneration of wild type, LIF knockout and *mdx* mice by acting on the population of muscle precursor cells to stimulate regeneration. LIF binds to components of the extracellular matrix [62], and this would allow LIF to

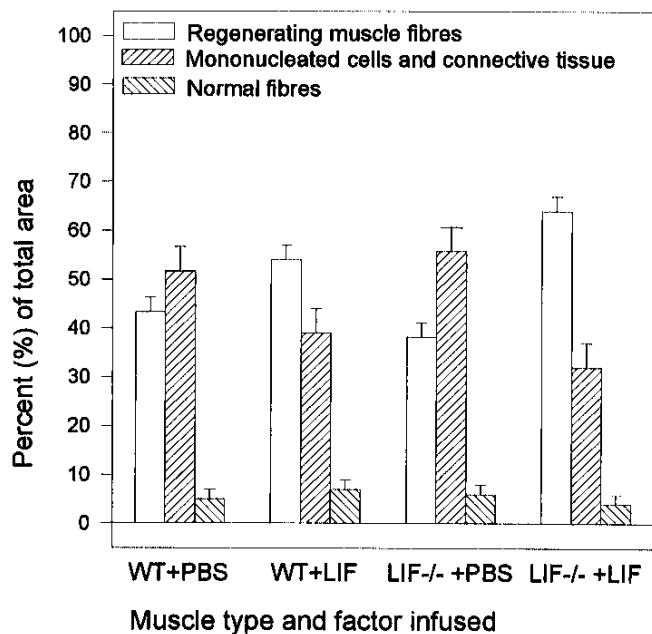


Figure 2 Volume density of regenerating muscle fibres after PBS and LIF treatment. Volume density $\pm$ SD in each group was determined and was defined as the area occupied by regenerating muscle fibres as a percentage of the total area measured. This was compared to the area occupied by mononuclear cells and connective tissue, and normal uninjured fibres.

Cytokines influencing regeneration of skeletal muscle

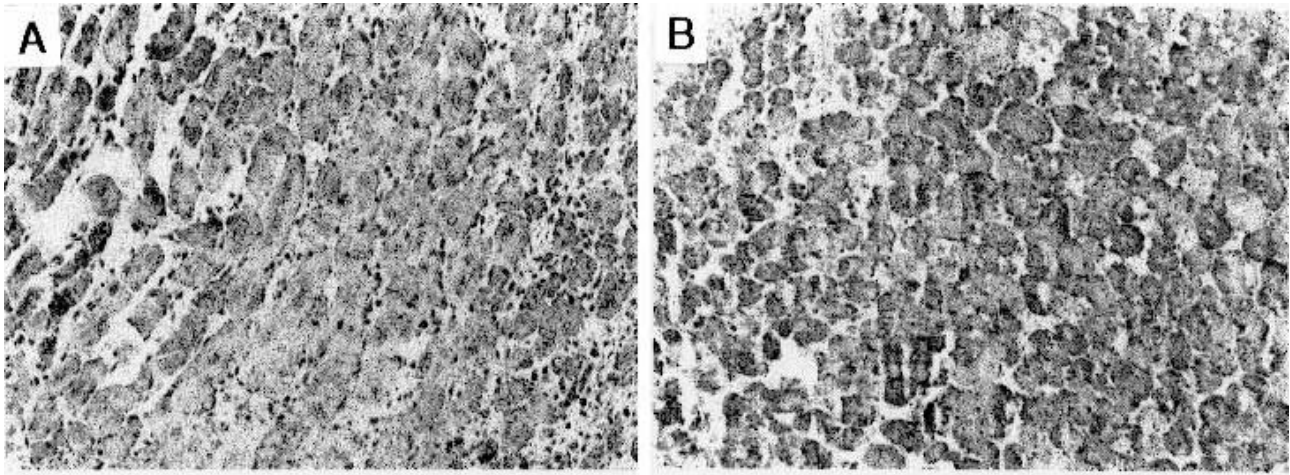


Figure 3. Desmin immuno-staining ($\times 200$) of wild type and LIF $-/-$ muscle 7 days after LIF treatment. Transverse sections (6 mm), were cut and stained for desmin, a muscle specific cytoskeletal protein. The sections were counterstained with haematoxylin. (A) Wild type and LIF; (B) LIF $-/-$ and LIF.

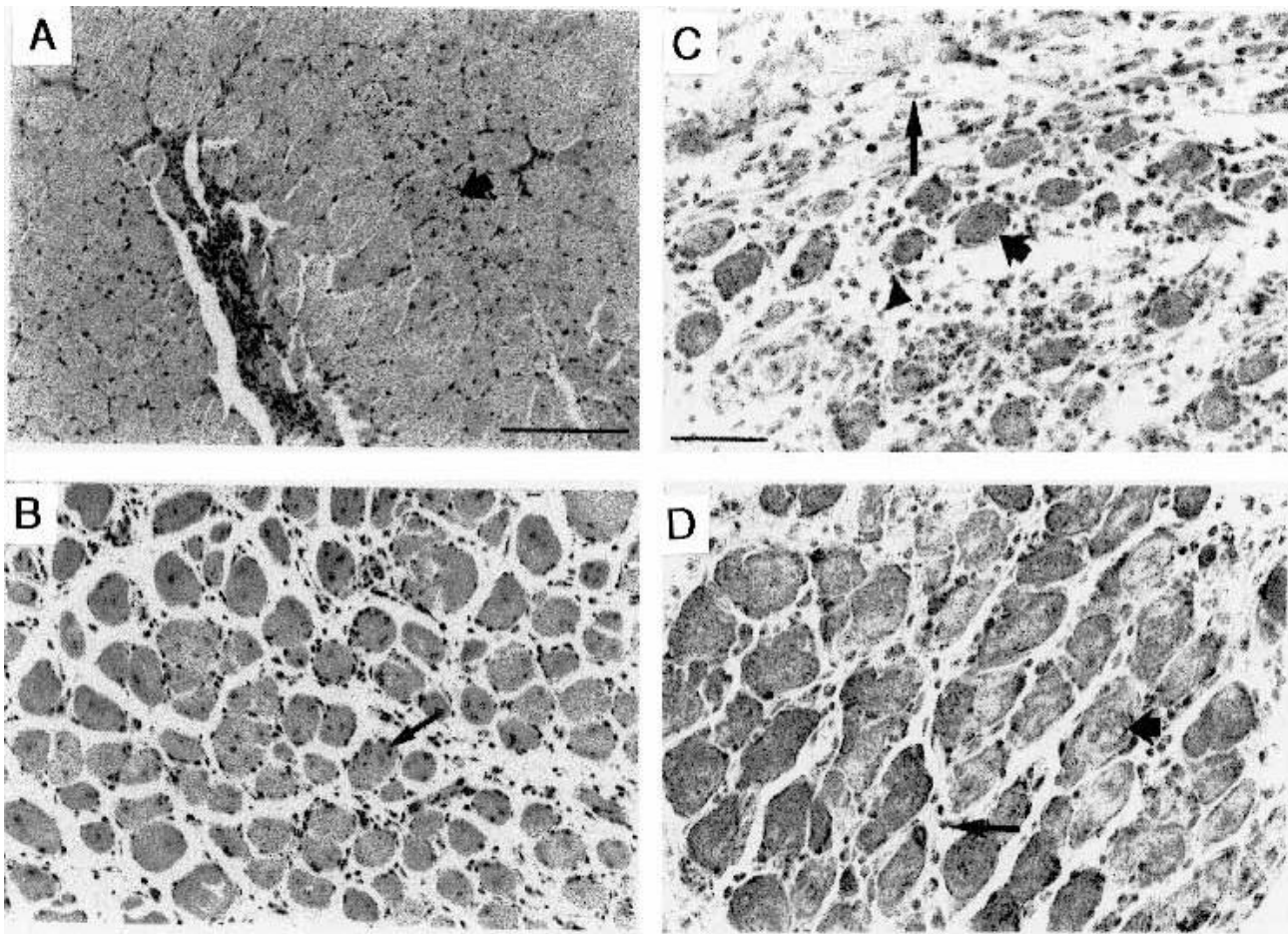


Figure 4. Cross section ($\times 200$) of the mdx vastus lateralis muscle before and after LIF treatment. Sections were stained with haematoxylin and eosin. Top left (A) mdx and PBS after 7 days; bottom left (B) mdx and LIF after 7 days; wide arrow indicates regenerating fibres; narrow arrow indicates LIF stimulated fibres. Bar: 100 μ m. Figure 4C & 4D. Desmin immuno-staining ($\times 400$) of mdx muscle 7 and 14 days after LIF treatment top right (C) mdx and LIF after 7 days, bottom right (D) mdx and LIF after 14 days. Wide arrow indicates stained fibre

Cytokines influencing regeneration of skeletal muscle

Table 3. Determination of average diameter of regenerating fibres in LIF treated mdx mice. LIF treated groups showed significant differences in fibre size after 7 ($p < 0.05$) and 14 days ($p < 0.01$) as determined using a two-tailed Student *t*-test. A minimum of 2500 fibres were measured in each group ($n = 4$ in each group).

Treatment	Average fibre diameter $\pm$ SD (mm)	
	Day 7	Day 14
PBS	24 $\pm$ 3.3	25 $\pm$ 1.0
LIF	31 $\pm$ 2.7	34 $\pm$ 2.4

remain locally in an active form for longer periods of time after infusion, rather than being dispersed via the circulation. The action of LIF in muscle is the first demonstration of an endogenous regeneration factor that acts *in vivo* after injury. This myotrophic action of LIF, together with its known neurotrophic action, makes LIF a potential therapeutic agent for the treatment of neuromuscular disease.

IL-6, TGF- α and bFGF Do Not Increase the Rate of Muscle Regeneration

Since IL-6 and TGF- α stimulate myoblast proliferation *in vitro*, the role of these two factors in muscle regeneration was examined. IL-6 (480 U/day) and TGF- α (24 ng/day) were applied exogenously to muscle for 7 days after injury using osmotic pumps. IL-6 and TGF- α did not stimulate regeneration when infused into the injured muscle. Table 1 shows that the average fibre diameter increased 12% for the IL-6 treated group and 6% for the TGF- α treated group, compared to the control group. However, perfusion of injured muscle with these factors did result in significant changes to the muscle. The histology showed that the perfused areas were occupied predominately by mononucleated cells and connective tissue [47]. There were only a few regenerating muscle fibres distributed within this area and Table 2 shows that there was a decrease in the fibre density. Immunohistochemistry showed that most of the mononuclear cells were nonmyogenic in origin, with very little specific desmin staining observed. The volume density (Fig 5) shows that 80% of the perfused area for the IL-6 treated group and 82% of the perfused area for the TGF- α treated group consisted of mononuclear cells and connective tissue, with less than 20% of each area designated as regenerating muscle fibres.

The results presented here show that IL-6 stimulated non-muscle cells, which would include fibroblasts which are associated with the muscle fibres. Radiolabelled IL-6 remained predominately in the injured muscle, so this indicates a direct *in vivo* role for IL-6 in muscle. This does not rule out the possibility that IL-6 stimulated myoblasts to divide, because new myotubes did form, but they were few and appeared to be overwhelmed by the

Table 4. Determination of regenerating fibres in mdx muscle before and after treatment with LIF. There is a significant increase in the number of regenerating fibres in the perfused area measured 14 days after treatment with LIF, when compared to the control groups. (* $P > 0.05$; # $P < 0.01$).

Treatment	No. regenerating fibres $\pm$ SD / 100 mm ² area	
	Day 7	Day 14
PBS	1.0 $\pm$ 0.01	0.68 $\pm$ 0.003
LIF	1.1 $\pm$ 0.01*	2.0 $\pm$ 0.1 [#]

non-muscle cells. This is in contrast to that observed with LIF. The *in vitro* data show that LIF did not stimulate muscle derived fibroblasts and this was reflected in the histology [47]. The role of IL-6 appears to be pleiotropic, with actions on both muscle and non muscle cells, and its role in muscle regeneration may be related to its involvement in immune reactions and acute phase responses.

The role of TGF- α may be more specific in terms of which cells are targeted in the muscle. TGF- α synthesis has been reported in activated macrophages [61] which are present in injured muscle during the early stages of regeneration. In addition, TGF- α is able to induce proliferation of other cell types that have EGF/TGF- α receptors, such as fibroblasts, which are intimately associated with muscle cells. Its role in muscle

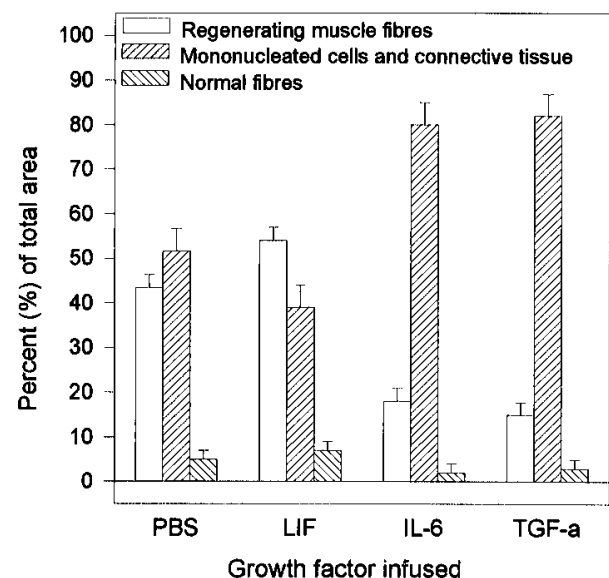


Figure 5 Volume density of regenerating muscle fibres after PBS, LIF, IL-6 and TGF- α treatment. Volume density $\pm$ SD in each group was determined and was defined as the area occupied by regenerating muscle fibres as a percentage of the total area measured. This was compared to the area occupied by mononuclear cells and connective tissue, and normal uninjured F1 fibres.

Cytokines influencing regeneration of skeletal muscle

regeneration may be indirect, stimulating nonmuscle cells, which would then influence the behaviour of muscle cells.

Despite the physiological role of FGF in the control of muscle cell proliferation and differentiation, exogenous application of bFGF to crush injured muscle of wild type mice has no effect on the rate of muscle regeneration [57]. A recent report has demonstrated that injection of neutralising antibodies against basic FGF into the muscle at the time of injury, reduced the size of the regenerating myofibres [50]. In *mdx* mice however, intramuscular injections of basic FGF does increase the number of regenerating fibres that are seen at the time of the first round of muscle necrosis-regeneration in 4 week old animals [51]. This would indicate that bFGF may be able to improve dystrophin deficient muscle regeneration. The precise role of basic FGF in muscle regeneration is still rather ambiguous.

We have carried out some preliminary experiments in which IGF-1 was perfused into injured muscle. The results suggest that there is no increase in muscle fibre numbers but there is some significant hypertrophy of both regenerating and normal muscle fibres.

Thus LIF is the only known growth factor, to date, which has been shown to be beneficial in the regenerative muscle process following injury and the inclusion of IGF-1 may have an additive effect.

Growth Factors and Muscle Regeneration: Past, Present and Future

Progress in understanding the early events of skeletal muscle regeneration was accelerated by the discovery of satellite cells [56]. Prior to this time, although the role of mononucleated myoblasts in regeneration was recognised, the origin of these cells was unknown. It is now clear from the work of many investigators over the past 30 years that satellite cells proliferate following muscle trauma and form new myofibres through a process equivalent to muscle histogenesis in the embryo. Extracellular stimuli play critical roles in the development and maintenance of the functioning neuromuscular system in all animals. In vertebrates a variety of soluble growth factors have been identified that have a profound effect on muscle cell physiology. Such factors can stimulate the proliferation and differentiation of myoblasts during development and trigger adaptive plasticity responses in the mature muscle after injury. The key feature of growth factors compared to classical hormones is that they are rarely found in the circulation but rather are produced by cells that are widespread in the body. Given the pleiotropy of cytokines, their local production and action may be a parsimonious way that the body can use the same cytokine/receptor system to effect multiple biological actions.

Identification of factors that may stimulate or inhibit muscle cell proliferation and differentiation has resulted almost exclusively from *in vitro* experiments with cultured satellite cells. Using culture procedures originally developed by Bischoff, cultures of satellite cells have been employed to demonstrate the mitogenic influence of factors such as bFGF, IGF-I and II and TGF- α on this myogenic cell type. The importance of growth factors in regulating the growth and differentiation of muscle cells is now widely recognised.

During the past decade, advances in the recombinant DNA technology have led directly to major advances in scientific understanding of pathological processes and in our ability to intervene in preventing the progression of disease. Application of this technology to neuromuscular disease is now occurring, and is yielding promising results. This review has focused upon the properties and activities of growth factors in skeletal muscle regeneration after injury and in disease. The results presented demonstrate that one of the most exciting new addition in recent years to the list of growth factors that influences muscle precursor cell behaviour is LIF. The mounting number of effects described for LIF in muscle and nerve suggest that LIF plays a role in the developing and regenerating neuromuscular system. This view is reinforced by data that shows that LIF expression is upregulated in response to muscle and nerve injury, and exogenous application of LIF after injury stimulates muscle regeneration. The evidence suggests that LIF *in vivo* may act as both a trauma and a survival factor.

The observed effects of LIF *in vitro* and *in vivo* demonstrate a role for LIF in muscle regeneration. The precise role that LIF and other factors have in the scheme of muscle development and regeneration are yet to be elucidated. Future studies examining the more intricate mechanisms employed by growth factors and coordinated by the cell will provide more complete models for muscle regeneration.

Targeted Delivery of Growth Factors: is This the Answer?

Drug delivery targeted to a discrete tissue or organ offers significant advantages over systemic administration. Drug delivery should ideally be confined to the target site, where it should reach adequate concentrations. It must also reach the target tissue at a concentration sufficient to elicit a therapeutic effect, despite obstacles like drug metabolism and permeability barriers. Delivering the drug directly to the target tissue can circumvent these barriers and improve efficacy. The use of a delivery system also overcomes protein delivery problems. The behaviour of recombinant proteins such as growth factors *in vivo* makes it difficult to demonstrate accurately their activity in animal models. Many growth factors have a

very short half-life *in vivo*, and when administered by conventional delivery methods, such as by injection, they are eliminated rapidly, resulting in wide variations in the levels of protein in the plasma and tissues. By limiting the distribution of protein to the target tissue, this method maintains effective levels of short half-life proteins, and minimises systemic side effects.

We have developed a controlled release system for LIF in which calcium alginate rods are formed, containing LIF at a predetermined concentration [8]. These rods release LIF at about 0.5% per day at a constant rate for at least 4 months. In addition there is no observable inflammatory response to the rod implants and following a period of 3 months in mice skeletal muscle, the rods remain wholly intact. This allows a set perfusion rate of LIF to be delivered to specific muscles.

Myoblast Transfer Therapy and LIF

Myoblast transfer therapy (MTT) has been trialled at a number of centres with little or no success. A major problem has been the inability of implanted myoblasts and their corresponding myotubes to survive post injection. We have examined whether the myoblast incorporation rate might be improved via the use of LIF [17, 72]. Myoblasts were grown under a variety of conditions and injected into the *tibialis anterior* muscle of 3-4 week old *mdx* mice, with various additions. Five injections of 1 ml each containing 2×10^5 myoblasts were used. Animals were killed one month later and the muscles analysed by two methods. Immunohistochemistry was used to localise dystrophin protein expression *in vivo* and a novel RT-PCR, Mae III digest method was employed to quantitate *mdx* and donor dystrophin mRNA [5]. When cells are only grown in the presence of LIF and injected, there is no increase in dystrophin expression. However, a single injection of LIF along with the cells induces a doubling of the levels of dystrophin in the muscle. When a controlled release of LIF is provided to the injected muscle, via an alginate: LIF rod releasing 0.5% per day, there was a further significant increase to some 5 fold over controls. These muscles were being perfused at rate of approximately 10 ng/day.

It is clear that LIF significantly increased the levels of dystrophin expression of both the mRNA and protein, even with a single application. The local LIF concentration under these circumstances would diminish with a biological half-life of around 1.5 hrs but the effects were noted one month later. This is most likely attributable to LIF acting to promote the incorporation or survival of donor myoblasts either by increasing the regenerative environment in the recipient muscle or by acting on the implanted myoblasts to facilitate incorporation. It is known that when LIF is applied to the repair site of a severed sciatic nerve there

is a significant sparing of gastrocnemius muscle atrophy lasting up to 12 weeks [23, 67]. Myoblast proliferation would not account for these findings. Continuous perfusion with LIF augments the result to a very significant degree. It has already been shown that when myoblasts are grown in bFGF there is also a significant increase in dystrophin positive fibres following MTT [46]. Thus a combined strategy of bFGF myoblast pre-treatment followed by *in vivo* LIF release to the injected muscle should provide even further augmentation.

LIF Treatment of Dystrophic *mdx* Muscle

The *mdx* mouse is a genetic model for the human Duchenne muscular dystrophy condition in that it lacks the protein dystrophin in its muscle. Its absence results in the de-stabilisation of the membrane, which causes permeations, and ion imbalances, which eventually result in muscle fibre necrosis. This breakdown initiates a pathway of regeneration in the muscle from precursor satellite cells as described earlier. This cycle, whilst initially in balance in the human, becomes inadequate resulting in the progressive loss of muscle fibres and replacement with connective tissue. The diaphragm muscle of the *mdx* mouse follows this same pathology as DMD muscle commencing from about 3 months of age. Our laboratory has commenced a study to look at the long term effects of LIF perfusion on the diaphragm muscle. We have established a technique to suture alginate rods releasing 10 ng of LIF per day, to one hemisphere of the *mdx* diaphragm, without any observable disruption to the animals normal functioning. The rods are attached for a period of 3 months after which they are removed and analysed by computer aided morphometric analysis. Parameters including fibre area, diameter and frequency will be analysed along with biochemical measurements of connective tissue levels using a hydroxyproline assay. These experiments are currently in progress but some preliminary results suggest that LIF is having a positive effect in slowing the diaphragm wastage process.

Growth Factor Treatment of Neuromuscular Disease

The ability of skeletal muscle to regenerate after injury is well established. Within the damaged muscle, a small population of mononuclear cells, the satellite cells, are activated in response to injury, proliferate, fuse into multinucleated regenerating myofibres, which express muscle-specific proteins, and mature to replace damaged fibres. The consecutive steps of muscle regeneration are modified and prevented from proceeding to completion in various neuromuscular diseases such as the muscular dystrophies. Duchenne muscular dystrophy, occurring in approximately 1 in 3500 live male births, is the most common and severe of the human muscular dystrophies. Primary approaches to

treatment have been many but emphasis has been placed on myoblast transfer therapy, which involves replacement of the missing gene product to its appropriate cellular compartment, and gene therapy, introducing a direct or truncated copy of the gene coding for dystrophin, the protein that manifests the deficiency. These approaches have had limited success due to high inefficiency, in terms of the amount of gene expression per sequence, even though once introduced into the muscle the stability of expression is high. Secondary therapies could be directed at the pharmacological compensation of the primary defect, either by replacing the function of the missing protein or by counteracting the disturbance caused by its absence and stimulating muscle regeneration.

Growth factors have a number of potentially important clinical implications, some of which may benefit patients with neuromuscular disease. The rationale for using trophic factors to treat neuromuscular disease includes the understanding that these molecules show some degree of selectivity for the population of cells in which they are effective.

Address correspondence to:

Dr. Lawrence Austin, Dept. of Clinical Neuroscience St. Vincent's Hospital, 41 Victoria Parade, Fitzroy VIC, 3065, Australia, phone (+613) 9288 3341, fax (+613) 9288 3350.

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Cytokines influencing regeneration of skeletal muscle

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