The Role of Growth Factors in Myoblast Transfer Therapy

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Abstract

Myoblast transfer therapy, despite unsatisfactory clinical trial results, is still a potential method for the rectification of primary muscle myopathies, provided the procedure can be significantly improved. The technique involves implanting donor myoblasts into diseased muscle by the injection of large numbers of these cells. The majority of transplanted myoblasts die and are rapidly removed following injection, which is believed to account for the low incorporation rates of donor myoblasts and the coincident short term expression of donor gene products. A major goal in this area of research is to enhance the levels of donor myoblast incorporation and consequently the duration of donor gene expression. Muscle growth factors are naturally occurring agents which have been shown to elicit strong actions on myoblast growth. The potential role for these factors, in particular, leukaemia inhibitory factor (LIF) and basic flbroblast growth factor (bFGF), in enhancing the efficacy of myoblast transfer therapy is discussed in this paper.

Key words: muscle, growth factors, myoblast transfer therapy, basic flbroblast growth factor, leukaemia inhibitory factor.

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The level of understanding of the effect of growth factors on myogenic cells *in vitro* has increased dramatically over the past few decades. As a result, a number of growth factor mediated effects have been identified including increased cellular viability in culture, an increase in the purity of myogenic cells, the induction of chemotactic responses, the enhancement of growth and fusion and the stimulation of *in vivo* regeneration.

Myoblast transfer therapy (MTT) is a method by which donor muscle cells are implanted into diseased host muscle to afford a rectification of the intrinsic genetic abnormality [58]. However, recent studies have identified a number of problems which must be addressed if MTT is to be a viable therapeutic approach in relevant clinical disorders. These are reviewed in detail in other chapters of this publication. Areas which have been shown to be amenable to growth factors is their use in a strategy to generate large numbers of fusion competent myoblasts and the improvement of *in vivo* efficacy of incorporation. Although the generation of large numbers of donor myoblasts is a requirement, it has been noted that the majority of injected cells rapidly die within 48 hours [23]. Various growth factors have been identified which activate myoblast proliferation and promote cell survival *in vitro* and in some instances enhance the process of muscle regeneration *in vivo.* These actions are of obvious benefit to myoblast transfer protocols by facilitating the generation of large numbers of myogenic cells for injection, promoting donor cell survival and by enhancing the process of regeneration thereby potentially increasing the incorporation of injected donor myoblasts into host myofibres.

In this paper we will briefly review myogenic agents which have demonstrated activity on myoblasts in vitro, and in more detail, present results using leukaemia inhibitory factor (LIF) and review the effects of basic flbroblast growth factor (bFGF) in myoblast transfer therapy.

Myogenic Growth Factors

With regard to the application of myogenic factors to myoblast transfer therapy there are many factors which have been shown to be myogenic *in vitro* and will be discussed in this context. Table 1 outlines some of the early reported effects of factors on the process of myoblast proliferation and fusion to form myotubes *in vitro.*

The identification of myogenic factors arose from the development of tissue culture methods for muscle tissue. Initial reports detailed the effect of growth hormone [39] testosterone [60], adrenocorticotropic-hormone [16, 17] and insulin [19] showing non-specific effects most likely due to a generalized anabolic influence as opposed to a direct stimulation of DNA synthesis. More recent reports

Table 1. Factors that influence the proliferative and differentiation characteristics of human, rat or murine skeletal muscle precursor cells (mpc) in vitro. t *indicates a positive influence;* ↓ *indicates a negative influence; - denotes where there is no demonstrated effect. ECM - Extracellular Matrix. The table is extended from information in Grounds and Yablonka-Reuveni [31]. The data on LIF, IL-6 and TGF-α are novel effects reported by ourselves.*

Factor	Proliferation	Fusion	Cell Type	Reference
Growth Factors				
Epidermal GF	t	-	mpc (human)	35
Fibroblast Growth Factor (basic)	t	↓	mpc (bovine)	29
Insulin-like GF-1 & II	t	t	L6 (rat)	25, 26
Insulin-like GF-I	-	t	mpc (rat)	1
Interferon	-	t	mpc (human)	24
Interleukin-6	t	-	mpc (murine)	5
Leukaemia Inhibitory Factor	t	-	mpc (murine)	5
Macrophage colony stimulating factor-1	t	-	mpc (murine)	43
Macrophage colony stimulating factor-1	t	-	murine C2	43
Platelet-derived GF (PDGF) (BB)	t	↓	murine (C2)	70
Transforming GF-a	t	↓	mpc (murine)	5, 6
Transforming GF-a	t	↓	mpc (human)	5, 6
Transforming GF-P	4	↓	murine C2	55
Transforming GF-P	↓	↓	mpc (rat)	1
Neuropeptides				
Adreno-corticotropin	t	-	mpc (murine)	17
Enkephalins	4	-	mpc (murine)	16
Other				
Bone morphogenic protein-2	-	4	L6 (rat)	72
Hemin	4	t	mpc (rat)	28
Prostaglandin El	-	t	mpc (human)	74
Transferrin	t	-	L6 (rat)	57
ECM Components				
Collagen	-	t	L6 (rat)	53
Fibronectin	t	4	L6 (rat)	59
Fibronectin	t	4	mpc (murine)	68
Laminin	t	-	mpc (rat)	27
N-acetylglycosamine	-	t	L6 (rat)	14

have detailed specific effects on muscle growth in vitro by factors such as basic fibroblast growth factor (bFGF), leukaemia inhibitory factor (LIF), insulin like growth factors I and II (IGF I & II), platelet derived growth factor (PDGF), interleukin 6 (IL-6) and transforming growth factor a (TGFa). Transforming growth factor p (TGFp), although a growth factor with significant effects on myoblasts in culture, will not be considered because its effects are largely inhibitory [36].

The Insulin-like growth factors (IGF-I & IGF-II) were first shown to influence muscle cells in culture in the late 1970's where studies were performed on both myoblasts and myotubes at different stages of development [22, 26]. Initial studies showed that IGF-I was capable of inducing a doubling in the growth rate of rat L6 myoblasts along with an increase in the rate of amino acid uptake. Subsequent studies showed that the response to the IGF's is dose dependent and biphasic. *In vivo*, IGF-I is increased in muscles during regeneration after ischaemic injury [42]. In addition it has been demonstrated that muscles undergoing active stretch induced hypertrophy strongly expressed two isoforms of IGF-1 which were produced by alternate splicing of the same gene [73].

It has been established that human growth hormone (GH) induces the production of IGF-1 in a number of tissues including muscle, liver, bone, cartilage and skin and in turn

IGF-1 levels act in a negative feedback manner to regulate pituitary production of GH. Consequently it is generally believed that many of the growth associated effects of GH are mediated via IGF-1. A number of human trials have been performed using GH alone or in combination with IGF-1. These are well reviewed in [52]. Observations in the treated groups included a significant increase in muscle protein synthesis and associated muscle mass, although associated increases in muscle function were less significant. Currently IGF-1, under the registered name of myotrophin, is awaiting FDA approval for use in the treatment of amyotrophic lateral sclerosis, with backing from the biotechnology company Cephalon Inc. (West Chester, PA, USA). Any potential clinical use of IGF-1 as an adjunct to myoblast transfer therapy would be aided by this approval, if granted, and consequently raises the profile of this agent as a potential candidate for the treatment of muscular dystrophy.

Platelet-derived growth factor (PDGF) has been shown to be a stimulator of skeletal muscle cell proliferation and a strong chemoattractant for these cells [70, 63]. These effects are dependent upon the particular isoform being studied, AA, AB or BB. Early studies using the AB isoform of PDGF showed no effect on muscle satellite cell growth [10]. Subsequent reports have shown that the BB isoform is a stimulator of muscle cell proliferation and that this isoform is the predominant species present in normal mouse and rat serum [70]. It has been proposed that PDGF serves a number of roles in muscle regeneration. Initially, release of factors such as PDGF may act as attracting agents for migratory myoblasts [63]. Once released it may then act as a mitogenic agent in conjunction with other factors to accumulate a large population of myoblasts for myofibre repair [32]. As yet no publications have arisen using this factor in models of *in vivo* muscle regeneration or MTT related work.

Interleukin 6 (IL-6) is an inflammatory cytokine which is expressed at low constitutive levels in normal muscle but the biological significance of this expression is not known [13]. It has been shown that IL-6 stimulates myoblast growth *in vitro* [5, 6]. This molecule is functionally related to the class of pluripotent factors, which amongst their properties have a neurotrophic action and share a common receptor component, the gp130 transmembrane signalling domain. Members of this family include LIF, ciliary neurotrophic factor (CNTF), oncostatin M (OM) and interleukin 11 (IL-11), [54]. We have demonstrated myogenic effects of both LIF and IL-6, while initial studies using CNTF *in vitro* showed no effect (unpublished observations). It has been shown that CNTF has myotrophic actions *in vivo* [38]. The perfusion of IL-6 into injured muscle *in vivo* has proved of no benefit in terms of muscle regeneration [48].

Transforming growth factor a (TGF-a) is implicated in skeletal muscle regeneration due to its positive effect on myoblast proliferation [5, 6]. Muscle cells express the TGF-a receptor (which is identical to the epidermal growth factor receptor) in culture (Bower, unpublished observations). However, TGF-a is inhibitory to myoblast differentiation *in vitro* and *in vivo* administration to crush injured muscle results in a deficit in muscle fibre formation, attributable to a loss in myofibre area [48]. Due to the similar activities of TGF-a with bFGF, in terms of timing of effect and morphological appearance of myoblasts, it would be interesting to pre-treat cultures of myoblasts with TGF-a prior to implantation (see next section on bFGF).

Basic fibroblast growth factor is a 16.4kD single chain polypeptide first isolated from pituitary extract [30] and was named for its ability to initiate DNA synthesis in cultures of Balb/c 3T3 fibroblasts in culture. There are nine members in this family which have a variety of actions such as growth, differentiation, angiogenesis, tissue repair and transformation [54]. Basic FGF or FGF-2, is the most studied in the muscle system, although it is likely that other members will have myogenic effects. The potent action of bFGF is seen over a wide range of concentrations as low as 0.01ng/ml. It is an ubiquitous molecule being found in a majority of all organs, tissues and cultured cells examined to date (Table 1). Most findings are based on *in vitro* data raising the questions as to whether the autocrine activities seen with bFGF are an adaptive response for artificial survival rather than a naturally occurring phenomenon [62].

bFGF in vitro

The myotrophic effects of bFGF have been studied in more detail than any other factor to date [37]. Gospodarowicz *et al* [29] were the first to describe a positive mitogenic effect of bFGF in bovine myogenic cells. They reported a ten fold increase in myoblast numbers in low serum as measured by ^{3}H thymidine incorporation. The same authors observed a decrease in myotube formation in the first 72 hours after addition of growth factor at a time when DNA synthesis was at its peak. This response declined with time and DNA synthesis accordingly decreased. Subsequent studies by other groups confirmed bFGF as a strong mitogen for myoblasts *in vitro* [2, 49, 56].

The effect of bFGF *in vitro* differs from that of a number of other myotrophic factors in that it induces a morphological change in primary derived myoblasts resulting in a more rounded and compact appearance as opposed to the characteristic elongated shape normally observed. The proliferative effect induced by a single stimulation with bFGF occurs early with a rapid rise and then plateau in cell numbers, occurring over some 2-3 days. Interestingly myoblasts from the *mdx* mouse have been shown to be more sensitive to the proliferative actions of bFGF than normal muscle cells, with optimal responses at 0.1-0.2 ng/ml for *mdx* and 1.0-2.0 ng/ml for normal myoblasts [20]. The role of bFGF in single fibre culture preparations has also been examined in a number of reports [11, 71]. These studies have revealed a more detailed picture of the behaviour of satellite cells in the presence of bFGF by showing that bFGF will initiate proliferation within a few

hours, however irrespective of its' continuing presence the majority of dividing cells will differentiate into myogenin positive muscle precursor cells. The authors propose the existence of an as yet unidentified factor which may sustain/inhibit satellite cells to maintain them in their proliferative state.

Basic FGF is classed as a heparin binding protein with a variety of truncated protein products displaying binding activity which indicates a number of heparin binding regions within the bFGF molecule [8]. It has been proposed that bFGF attaches to the extracellular matrix components in tissue where it can be localised to act on discrete populations of cells. Indeed the accumulation of inflammatory cells at a damaged site in muscle will initiate the enzymatic release of heparin bound bFGF. These observations and the fact that bFGF has been shown to be angiogenic have led to the suggestion that bFGF is a general tissue repair factor [32].

bFGF in vivo

Basic FGF is one of many factors which are synthesised and released following muscle damage where they may potentially activate satellite cells and stimulate myoblast proliferation and ultimately contribute to muscle repair. Studies have shown that dystrophic mice including the *mdx* mouse have elevated levels of bFGF production [3]. Despite this, studies to date show that the exogenous addition of bFGF, both by intramuscular injection and slow release from polymers, to an injured muscle does not result in any significant increase in new muscle formation [50]. This does not mean that endogenous bFGF is not involved in the regenerative process but that the further addition of bFGF has no real benefit. This does however suggest that the administration of bFGF *in vivo* as an adjunct to myoblast transfer will be of no benefit.

bFGF increases the efficacy of myoblast transfer

Kinoshita and co-workers [44] have shown that the preincubation of myoblasts with bFGF increases the efficiency with which donor myoblasts are incorporated into immunosuppressed host muscles of the *mdx* mouse. Donor myoblasts were incubated in bFGF for 48 hours prior to injection and the average percentage of hybrid fibres observed after 28 days was 34.4%. They reported that in some cases this figure was as high as 40%, as measured by β-galactosidase reporter gene expression and immunostaining of wild type dystrophin. These positive effects were observed over a range of bFGF concentrations, ranging from 10 ng/ml to 100 ng/ml. The beneficial effect of incubating cells in the presence of bFGF may be attributable to an advantage afforded to them by increasing their long-term viability or their capacity for proliferation, thus making available a larger pool of donor myoblasts which may fuse with host myofibres.

When bFGF treated cells were injected in two successive injections seven days apart the percentage of hybrid fibres again increased to over 50%. This represents the most efficient incorporation of donor myoblasts into host myofibres reported thus far, apart from studies where muscles have been previously irradiated to reduce the influence of resident immunological cells or damaged by injection of notexin to induce a new cycle of regeneration in hosts. The high percentage of hybrid fibres observed after a second set of injections could be attributed to damage caused by these injections which may trigger a new cycle of regeneration. Thus any cells surviving from the original injections would be immediately available for fusion. Alternatively, successive injections introduces a greater number of myoblasts into a particular muscle, thus overcoming technical limitations where the numbers of cells which can be adequately resuspended is finite as is the volume of liquid which can be injected into the muscle without resulting in leakage of cells and disruption of muscle architecture. The large numbers of cells introduced may also result in an increased diffusion of myoblasts throughout the muscle.

This increase was achieved despite *in vitro* observations that bFGF inhibits differentiation and fusion of myoblasts [1, 20]. However this inhibition is reversible since withdrawing bFGF following a 48 hour exposure allows myoblasts to regain their ability to fuse [44]. An explanation which may account for some or all of the increased donor survival effects is the potential induction of metalloproteases which can degrade cell matrix components such as those in the basal lamina. This could increase the mobility of injected myoblasts within the extracellular space thereby increasing the frequency with which donor myoblasts fuse to host myofibres. It should be noted however that the observations on migration of implanted myoblasts were made either with permanent cell lines or after long periods (250 days) of time and only in irradiated muscle [51, 69]. More recent reports suggest there is a limited amount of diffusion after 30 days [61, 69].

It is interesting to note that if bFGF is injected into the muscle in combination with myoblasts there is no net increase in the number of hybrid fibres observed [44]. There may be a number of reasons for this. It may be that bFGF inhibits the fusion of donor cells as it has been shown to inhibit myotube formation *in vitro*. Basic FGF is endogenously produced at high levels within *mdx* muscle and the addition of exogenous bFGF may not increase the efficiency of muscle repair. The injection of bFGF directly into the muscle may also counteract any benefit of induction within donor myoblasts by acting on resident host cells such as satellite cells.

While the number of hybrid fibres obtained with injection of bFGF treated myoblasts does not approach the 80-90% incorporation achieved in other studies, these results are clinically more relevant as they were achieved without prior treatment of the muscle with radiation, injection of notexin or within immuno-compromised hosts. The conditions used in this series of studies mirrors to a greater extent the conditions under which myoblast transfer therapy would foreseeably be used in future clinical trials, as

techniques such as irradiation or notexin mediated damage would present further obstacles.

Leukemia inhibitoryfactor (LIF) is a multipotent cytokine inducing a wide range of effects in a wider range of cells types. LIF belongs to a family of cytokines with structural and functional similarities including IL-6, interleukin 11 (IL-11), oncostatin M (OM) and ciliary neurotrophic factor (CNTF). LIF has a wide range of effects on many different cell types including myoblasts, haemopoietic cells, embryonic stem cells, primitive germline cells, hepatocytes, neurons, adipocytes and osteoblasts [41]. The effect LIF has on cells varies with the cell type. The effects mediated by LIF range from stimulation of myoblast proliferation [5] to induction of differentiation and colony formation in murine leukemia (M1) cells [31]. The mature LIF protein is basic and heavily glycosylated mainly at asparagine residues. Variation in the extent to which the protein is glycosylated accounts for observed variations in mass and charge (Mr : 32,000 - 67,000 ; pI 9). The extent to which LIF is glycosylated does not effect *in vitro* function, as the function of both E.Coli produced non-glycosylated LIF and hyper-glycosylated LIF produced in yeast does not differ from the natural product.

LIF in vitro

LIF is amongst the most potent myogenic agents described *in vitro.* Studies by us have shown that under identical conditions, the response of myoblasts to LIF is greater than that observed with other myogenic factors such as bFGF, PDGF, IGF-I, IL-6 and TGF-a [5, 6]. Unlike some of the other factors, the response of myoblasts to LIF, in terms of cell numbers, is long term with a significant increase in numbers not seen until four to six days after growth factor addition. This effect is most notable when fetal calf serum levels are sub-optimal, suggesting a survival or indirect role for this factor. When LIF is used *in vitro* in combination with bFGF or TGF-a the effect on proliferation is totally additive, exceeding that normally seen with either factor alone [6].

The in vitro effect of LIF on myoblasts is mediated through specific cell surface receptors. The binding characteristics of these receptors differs from those detected on other cell types such as embryonic stem cells, hepatocytes and macrophages with an intermediate class of receptor being identified on myoblasts with a Kd of 400pM and approximately 20,000 binding sites per cell when cultures are at ~50% of confluence [12]. Down regulation of these receptors is observed as the *in vitro* density increases, upon fusion of myoblasts to form myotubes, or after pre-treatment of the cells with LIF indicating the presence of feed-back regulation of the receptor. After the binding of LIF to its receptor it is internalized and subsequently degraded. Reports based on other cell types show that LIF is internalized along with its receptor [40].

Other myogenic factors such as bFGF, PDGF and TGF-P suppress and in some cases completely inhibit the in vitro fusion of myoblasts [32]. More recent evidence suggests that this effect is not irreversible. These observations are in direct contrast to similar experiments conducted with LIF which show no effect of LIF on the number of myotubes formed [67]. LIF does not alter the expression of myogenic differentiation markers such as the contractile protein myosin or the acetylcholine receptor. While LIF does not increase the number of myotubes formed *in vitro* it does increase the number of nuclei per length of myotube, which may be a reflection of the denser population of myoblasts prior to fusion following incubation with LIF (Bower, unpublished observations). However, experiments where myoblasts are allowed to reach a higher density without prior growth factor treatment do not reflect this. The number of myotubes is increased but the number of nuclei per length of myotube does not change. This may indicate that LIF has a hypertrophic effect on myotube formation inducing the formation of larger myotubes. These observations are reflected by the fact that myoblasts incubated with LIF do not lose their ability to fuse with host *mdx* myofibres after injection (discussed later in this section).

LIF in vivo

The most advantageous myogenic effect of LIF in terms of myoblast transfer therapy is the enhanced level of regeneration observed *in vivo* following the perfusion of LIF into a damaged muscle [9, 48]. Other groups have also reported myotrophic actions of LIF on denervated muscles [38, 21]. This potent effect of LIF on the efficiency of muscle regeneration suggests that the application of LIF, in combination with myoblast injections, may offer a benefit to myoblast transfer therapy.

LIF is produced in wild type C57BL10\ScSn muscle after crush injury and by *mdx* muscle undergoing pathological regeneration [46]. LIF has been shown to be produced by infiltrating macrophages which serves a dual purpose in that it is myogenic for muscle cells and is chemotactic for myoblasts. LIF mRNA is produced within 3 hours of crush injury and levels remain elevated for 24 hours after which it decreases to an intermediate level which is maintained for the next ten to twelve days. The pattern of expression of LIF mRNA is consistent with the temporal pattern of muscle precursor cell proliferation in regenerating muscle where the numbers of replicating myoblasts is increased from about 24 hours post injury to a peak at 72 hours remaining high for the next five to seven days [33].

Studies conducted using the LIF knockout mouse (C57BL6J LIF -/-), an animal first described by Stewart *et al.* [65], demonstrate a reduced capacity for regeneration after crush injury, as measured by morphometric analysis [48]. The diameter of regenerating fibres from LIF (-/-) mice were 25% smaller than those from wild type control LIF (C57B16J LIF +/-) animals seven days after crush injury. When exogenous LIF was perfused via an osmotic pump to the crushed muscle of LIF (-/-) and control (+/-) muscle the average fibre diameter increased by 153% in wild type muscle and 193% in LIF (-/-) muscle over control groups ($p > 0.001$). A corresponding increase in volume density in LIF treated muscle was also observed, increas-

ing from 43 to 54% of total regenerating area in wild type muscle and from 38 to 64% in LIF -/- muscle ($p > 0.05$).

More importantly, in terms of myoblast transfer therapy LIF has a positive effect on the regenerative capacity of *mdx* muscle. After seven days of continuous infusion of LIF into uninjured *mdx* muscle the muscle fibre diameter increased by 22% compared to control groups [47]. Histological examination revealed an increased number of mononucleated cells which anti-desmin staining revealed to be largely myogenic. The remainder of the unstained mononuclear cells appeared morphologically to be infiltrating polymorphonuclear leukocytes and macrophages. The presence of these cells is not solely due to focal injury caused by insertion of the osmotic pump cannula as control animals infused with saline did not exhibit this increased population. It is more likely that LIF, by direct or indirect means, induced the accumulation of these cells.

The effect of LIF was sustained as animals which were examined seven days after LIF infusion had ceased exhibited an increased muscle fibre density and fibre diameter some 30% larger than control animals. After 14 days the number of mononucleated cells decreased with the associated increase in fibre diameter and density suggesting that many of the myogenic mononucleated cells had fused to form myofibres.

Infusion of ^{125}I-LIF into injured muscle by an implanted osmotic pump over 24 hours showed localisation and retention of the majority of the infused LIF (78%) within the muscle and more particularly the damaged (17%) and distal regions (61%) of the muscle, as determined by γ counts of tissues [9]. Levels in other tissues confirmed that by far the majority of the infused ^{125}I-LIF was retained within the muscle, while excretory routes showed low levels of LIF; liver (1.8%), kidney (1.8%) and blood (0.4%). This observation suggests that LIF is retained in the muscle possibly by binding to a component of the extracellular matrix. A large proportion of the infused LIF remained viable and was not degraded as indicated by the relatively low levels of ^{125}I-LIF in the thyroid (0.008%). This observation was confirmed by experiments utilising an alginate based slow release system for LIF [7] which indicates that LIF is primarily retained in the adjacent muscle to the implant with only trace amounts detected in other tissues. Direct injections of LIF into mouse skeletal muscle have shown that the biological half-life of LIF in muscle is 1 1/4 hours (Austin, unpublished observations).

The exogenous addition of LIF into an injured muscle results in a decrease in the area occupied by connective tissue and a coinciding increase in the volume density of regenerating fibres at five days post injury. The fibroblast is the primary cell component of connective tissue, and while it produces LIF mRNA [66] it does not respond in a growth related manner *in vitro*. It may be that there are autocrine effects of LIF on flbroblasts which result in the changes in connective tissue levels.

Other evidence which supports the *in vivo* effect of LIF in muscle shows that subcutaneous injection of LIF rescues

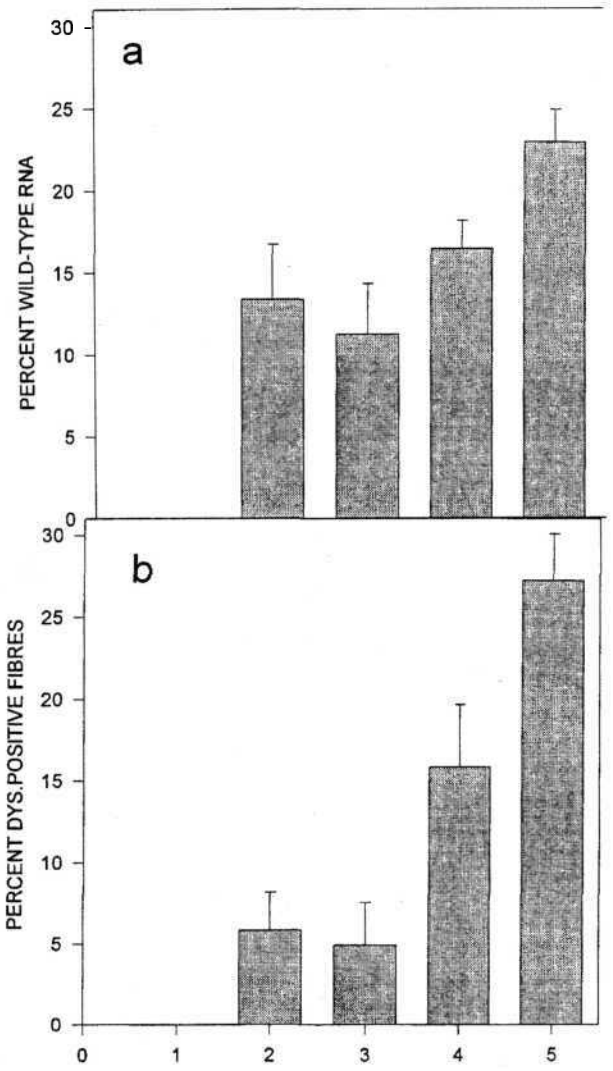

Figure 1. (A) Densitometric analysis of PCR products showing the percentage of wild type dystrophin mRNA expressed in mdx tibialis anterior 28 days after injection of 2 x 10^6 wild type C57BL10\ScSn myoblasts. Error bars indicate range (n = 3). 1 - mdx muscle saline injected. 2 - Injection of control cells (no growth factor). 3 - Injection of cells incubated in LIF for 48 hours. 4 - Injection of cells incubated in LIF for 48 hours with 30U/ml mLIF in the injection carrier solution. 5 - Injection of cells incubated in LIF for 48 hours with LIF releasing rods adjacent to muscle. (B) Dystrophin positive cells expressed as a percentage of total muscle cells. These cells were assessed as described in the text. Columns as for 1-5 above. (n = 9). Error bars indicate SD.

skeletal muscle from denervation-induced atrophy [18, 21]. Denervated muscles rapidly upregulate mRNA for LIF receptor components, suggesting further that there is a direct myogenic effect of LIF on skeletal muscle. LIF mRNA is increased in the gastrocnemius muscle 24 hours after denervation by sciatic nerve transection peaking after

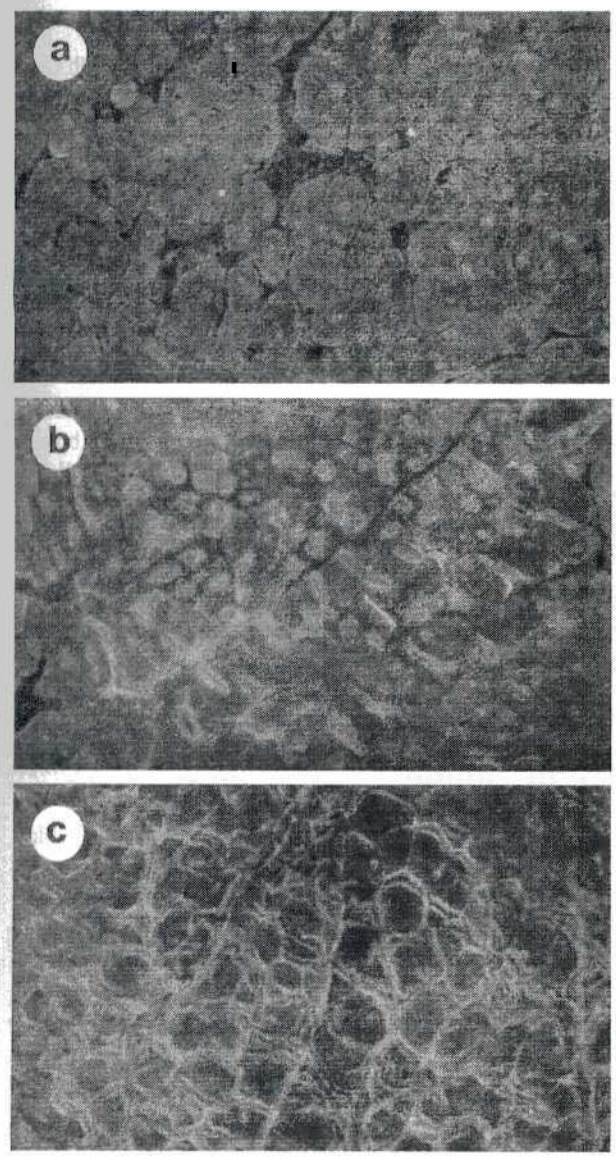

Figure 2. Photographs depict FITC double-labelling of dystrophin at x100 magnification. (A) mdx muscle injected with saline (procedural control). (B) mdx muscle injected with control cells (untreated). (C) mdx muscle injected with myoblasts grown in the presence of 30U/ml LIF and exposed to a slow release of LIF via alginate:LIF implanted rods.

seven days and declining to constitutive levels 14 days later [46].

Enhancement of myoblast transfer by LIF

We have injected myoblasts derived from the skeletal muscle of C57Bl10 mice into the left and right tibialis anterior muscles of the *mdx* mouse. The injections consisted of a total of $2x10^6$ myoblasts per muscle, in the presence and absence of LIF. Cells were grown in the presence of LIF prior to injection and, in other instances, LIF was injected with the myoblasts, or controlled release alginate rods containing LIF [7] were implanted adjacent

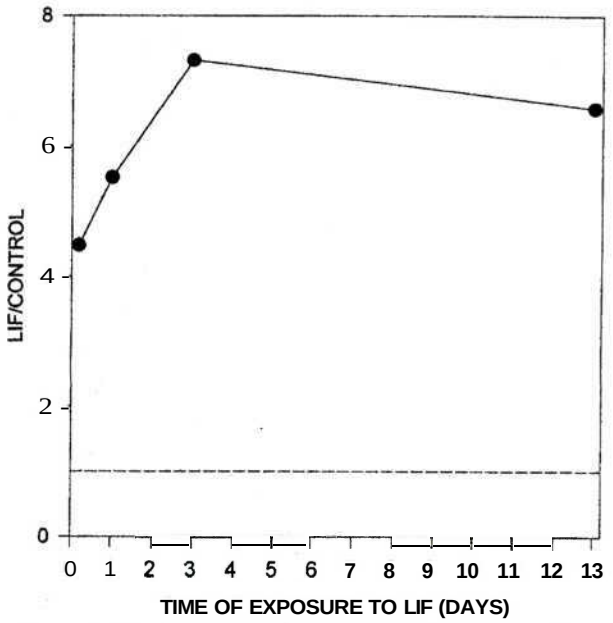

Figure 3. Myoblast proliferation, at 13 days in culture, shown as LIF/control ratio after exposure of cells to LIF for various periods of time. The first point is an exposure of 4 hours. Cells were grown in the presence of LIF for the times shown, washed free of LIF and growth was continued for 13 days. The responses show that an exposure for as little as 4 hours significantly enhances the final degree of proliferation.

to the muscle. The rods were developed to release about 5ng of LIF per day. All mice were left for four weeks and then the injected muscles removed. The dystrophin mRNA was amplified by RT-PCR in 30 cycles of replication, using specific primers which were designed to amplify a region of the murine dystrophin gene encompassing the *mdx* point mutation [4]. Restriction enzyme digest with the Mae III enzyme exhibits different sized products for wild type and *mdx* dystrophin cDNA. The products were resolved using polacrylamide gel electrophoresis on 12% gels and autoradiographed using Biomax film (Kodak). The left muscle was homogenised and total RNA extracted by standard methods [15]. The right muscle was frozen, sectioned and stained by indirect antibody immunolocalisation. The primary antibody was polyclonal sheep anti-dystrophin [45] and the secondary was rabbit anti-sheep conjugated to HRP and visualised by DAB/H_2 O2 staining. The sections were then examined in a blinded manner, for the presence of dystrophin expressing fibres. Three regions within the middle two thirds of the muscle were counted from at least 2 different animals. A quantitative analysis of the mRNA expression levels for dystrophin as determined by densitometric scans using Sigmagel (Jandel Scientific) is shown in Figure 1a. The proportions of positive fibres from each treatment group are shown in Figure 1b. FITC stained sections *of mdx* muscle before and after

myoblast implantation with and without LIF releasing alginate rods are shown in Figure 2.

It can be seen from Figure 1 that when allogenic myoblasts are grown in the presence of LIF and injected into *mdx* muscle, unlike bFGF, there is no increase in the levels of fusion as determined by dystrophin expression. However the co-administration of LIF with injected myoblasts results in an enhanced level of dystrophin expression in the muscle at 4 weeks, presumably due to an increased incorporation of donor cells. Animals were also injected with cells not grown in LIF but with LIF in the carrier solution only and these gave similar results (results not shown). This effect is increased still further following the sustained release of LIF via the alginate controlled release implants where a four fold increase over controls is observed. It can be seen that there is reasonable agreement between the two methods for determination of dystrophin expression.

As stated above, LIF has a biological half-life of 1 ¾ hours in muscle. Furthermore, previous results have shown that a relatively brief exposure (4 hours) of LIF to myoblasts is required to mediate growth responses in culture as observed at 12 days [6]. Figure 3 has been adapted from those results and demonstrates that a 4 hour exposure and withdrawal will result in a 5 fold rate of myoblast growth in culture. Thus repeated injections of LIF for a period after myoblast injection should significantly enhance the development and fusion of dystrophin positive myoblasts.

The individual studies using LIF *in vivo* and bFGF *in vitro* have demonstrated a capacity for these agents to enhance the mechanisms of myoblast transplantation by increasing the efficiency of myoblast fusion in the *mdx* mouse. The actions are clearly independent and it remains to be seen whether combined treatment regimes will further improve expression levels of dystrophin. In addition, these studies have been conducted over a short time interval of 1 month. Future studies will need to address the long term effects looking at periods of 6-12 months to see whether expression can be maintained. Encouraging results would warrant trials in a bigger animal model with associated clinical deficits, such as the golden retriever x-linked muscular dystrophy animals of Joe Kornegay (USA), in order to test for clinical benefits.

In addition, there is a long term requirement for studies directed at understanding the mechanisms by which LIF and bFGF afford a benefit to muscle cell transplantation. The resolution of the molecular interactions involved in these systems should provide muscle researchers with powerful tools for the design and implementation of treatments for muscle diseases.

In summary, the use of bFGF during the culture of myoblasts for implantation and the presence of LIF for a time after implantation, could have substantial clinical benefits in any future clinical trials of myoblast transfer therapy.

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