Low-energy laser irradiation promotes the survival and cell cycle entry of skeletal muscle satellite cells

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Accepted 8 January 2002

Summary

Low energy laser irradiation (LELI) has been shown to promote skeletal muscle cell activation and proliferation in primary cultures of satellite cells as well as in myogenic cell lines. Here, we have extended these studies to isolated myofibers. These constitute the minimum viable functional unit of the skeletal muscle, thus providing a close model of in vivo regeneration of muscle tissue. We show that LELI stimulates cell cycle entry and the accumulation of satellite cells around isolated single fibers grown under serum-free conditions. These effects act synergistically with the addition of serum. Moreover, for the first time we show that LELI promotes the survival of fibers and their adjacent cells, as well as cultured myogenic cells, under serum-free conditions that normally lead to apoptosis. In both systems, expression of the anti-apoptotic protein Bcl-2 was markedly increased, whereas expression of the pro-apoptotic protein BAX was reduced. In culture, these changes were accompanied by a reduction in the expression of p53 and the cyclin-dependent kinase inhibitor p21, reflecting the small decrease in viable cells 24 hours after irradiation. These findings implicate regulation of these factors as part of the protective role of LELI against apoptosis. Taken together, our findings are of critical importance in attempts to improve muscle regeneration following injury.

Key words: Satellite cells, Laser irradiation, Apoptosis, Myofiber, Proliferation

Introduction

Normal skeletal muscle contains post-mitotic muscle fibers that themselves have very limited remodeling capability. Remodeling is largely accomplished by myogenic cells derived from quiescent mononuclear satellite cells, which lie beneath the basement membrane of the muscle fiber, but outside of the plasmalemma of the multinucleated fiber itself (Mauro, 1961). Satellite cells are quiescent in normal adult mouse muscle (Schultz et al., 1978). They do, however, replicate in growing postnatal muscle, where they add myonuclei to enlarging muscle fibers, and in regenerating adult muscle, where they give rise to new muscle fiber segments to replace those lost during muscle injury (reviewed in Bischoff, 1994).

Mechanisms of muscle fiber repair are of interest in understanding the restoration of muscle after trauma or injury and during myopathic diseases. After severe injury, muscle regeneration is a slow process, during which scar tissue formation competes spatially with the regenerating muscle fibers at the site of injury. Many approaches have been suggested to enhance muscle restoration, including the use of muscle precursor cells, stem cells or muscle fiber transplants (reviewed in Grounds, 1999). One of the disadvantages of cell transplantation methods is that most donor cells fail to survive (Rando et al., 1995; Fan et al., 1996; Beauchamp et al., 1997). Involvement of the host immune system in this rapid death has been suggested (reviewed in Smythe et al., 2000), but the precise mechanism or mechanisms underlying the phenomenon remain obscure.

Low-energy laser irradiation (LELI) has been found to modulate various biological processes (reviewed in Conlan et al., 1996; Karu, 1999), such as increasing mitochondrial respiration and ATP synthesis (Morimoto et al., 1994; Yu et al., 1987), facilitating wound healing (Conlan et al., 1996) and promoting the process of regeneration and angiogenesis (Weiss and Oron, 1992; Bibikova and Oron, 1993; Bibikova and Oron, 1994; Bibikova et al., 1994). The augmentation of regeneration by LELI has been studied in various tissues, such as skin (Conlan et al., 1996), bone (Yaakobi et al., 1996), nerve (Assia et al., 1989) and skeletal muscle (Weiss and Oron, 1992; Bibikova and Oron, 1993). In the skeletal muscle of rats and toads, He-Ne laser irradiation of the injured site enhanced regeneration by two- and eightfold, respectively, relative to non-irradiated controls (Weiss and Oron, 1992; Bibikova and Oron, 1993). The injured site in the LELI-treated animals featured many young myofibers, suggesting that satellite cells are the major irradiation-responsive candidates (Weiss and Oron, 1992). This idea is supported by studies of the effect of LELI on primary rat satellite cell cultures, revealing the induction of cell cycle regulatory protein expression, increased satellite cell proliferation and inhibition of cell differentiation (Ben-Dov et al., 1999). In the search for a mechanism by which LELI manifests these cell responses, we very recently demonstrated that LELI specifically activates the mitogen-activated protein kinase/extracellular signal-regulated protein kinase (MAPK/ERK) pathway, but not the stress pathways (i.e. p38 MAPK and the stress-activated protein kinase/Jun N-terminal kinase
in a 24-well Primaria tissue culture pre-coated with 1 mg/ml growth medium were separated from the whole muscle by repeated gentle pipetting. The left and right EDL muscles were carefully removed and transferred to a plastic Petri dish containing DMEM, and muscle fibers and myogenic cultured cells were then mounted with fluorescent mount medium (Dako) followed by Alexa-594-conjugated streptavidin (1:500 dilution; Molecular Probes). The stained samples were viewed with a fluorescent microscope. Nuclei were detected with 46-diamino-2-phenylindole (DAPI; Sigma-Aldrich).

In the present study, the effects of LELI on cell survival and proliferation were tested in a single fiber system representing a three-dimensional organization of the surviving and repaired segments of the fiber (Rosenblatt et al., 1995). This serves as a more comprehensive model than standard tissue culture in which to assess satellite cell proliferation. We show that LELI promotes the cell cycle entry and accumulation of satellite cells around fibers grown under serum starvation and that these effects are synergistic with serum addition. The inhibitory effect of LELI on cell apoptosis goes hand in hand with increasing Bcl-2 and decreasing BAX expression in both these fibers and myogenic cultured cells.
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apoptotic only when they had pyknotic and/or fragmented nuclei
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1999; Shefer et al., 2001). Therefore, in the first set of experiments
we used isolated single fibers kept in serum-free medium for the entire experiment. Single myofibers were
irradiated 19 hours post plating, and the number of cells
emanating from each fiber was monitored daily for 5 days
following irradiation. Previously, it was shown that these cells
are virtually all myogenic and when grown in growth medium
have the ability to re-enter the cell cycle (Rosenblatt et al.,
1995). At zero time, virtually all the cells were still attached
to the fibers (data not shown); 1 day post irradiation, the average
number of cells adjacent to the LELI fibers was approximately
twofold higher than that in controls (see further on).

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fibers suggested an immediate effect of LELI on the cell cycle
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LELI activates quiescent satellite cells and increases their
proliferation (Ben-Dov et al., 1999; Shefer et al., 2001). To
evaluate the state of cells in the S-phase, BrdU was applied
immediately after irradiation, and fibers were fixed and
immunostained for BrdU after 1 day. BrdU incorporation could
be clearly seen in cells that were still attached to the LELI-
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Cell viability and apoptosis assays
Cell viability was assessed by the MTT (3-(4,5-dimethylthiazol-2-yl)-
2,5-diphenyltetrazolium bromide; Sigma-Aldrich) assay for
mitochondrial activity. At the indicated times, MTT was added to the
cells at a final concentration of 0.5 mg/ml for 2 hours of incubation.
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were then fixed with 2% paraformaldehyde, washed in PBS and
visualized under a fluorescence microscope. Cells were scored as
apoptotic only when they had pyknotic and/or fragmented nuclei
(Stadelmann and Lassmann, 2000).

Statistics

Unless otherwise indicated, raw data were analyzed using one- or two-
way analysis of variance (ANOVA). In within-group tests, when data
significantly deviated from the normal distribution (Kolmogorov-
Smirnov test for normality), a non-parametric t-test was carried out.
Alpha level was set to 0.05 in all experiments.

Fig. 1. LELI promotes cell cycle entry of cells attached to single
fibers. BrdU was added to control non-irradiated (A) or LELI (B)
fibers immediately after irradiation for 1 day, after which they were
fixed and examined for BrdU incorporation by
immunohistochemistry. Most of the cells in the non-irradiated fibers
were negative for BrdU, whereas some were positive (brown) in the
LELI fibers. Arrows indicate some of the BrdU-positive cells. Bar,
30 µM.

LELI increases the number of fiber-adjacent cells under serum-free conditions

We next analyzed the kinetics of cell accumulation near
irradiated and non-irradiated fibers. Four replicates of the
serum-starvation experiments were carried out, and statistical
analysis did not reveal any significant difference in the counts
of cells adjacent to fibers (Kruskal-Wallis ANOVA median test;
R (4,12)=3.00; P=0.0626; n.s.). Therefore, the results of these
four replicate experiments were pooled and averaged. The
cumulative plot of the number of cells per fiber clearly shows
that as early as day 1 (i.e., 1 day post irradiation), the irradiated
fibers produced more cells than did the non-irradiated fibers
(Fig. 2). The average number of cells per fiber was twofold
higher in the LELI fibers (P<0.001). A similar pattern of
distribution was accompanied by an increase in the average
number of cells per fiber, which reached more than threefold
the control group values, suggesting that LELI enables cell
proliferation (Fig. 2, right panel; Fig. 1). However, overall
proliferation was not pronounced and cell number per fiber was
even reduced on day 5 (Fig. 2, right panel), suggesting that
LELI is necessary but not sufficient to promote cell
proliferation for long periods under serum-free conditions and
that additional factors are required.

Synergistic effect of LELI and serum on cell accumulation near the fibers

To test the relationship between LELI and serum, we
conducted similar types of experiment but now with fibers kept
in low-serum-containing medium (0.1% HS). In general, on
day 1 fibers of both control and LELI groups already had
relatively high numbers of adjacent cells, probably owing to the
presence of the serum (Fig. 3A). However, in the case of
the control fibers, cell accumulation increased only slightly on
day 2 and average cell number per fiber remained almost the
same during subsequent days (Fig. 3A, left panel). In contrast,
in the LELI fibers, a noticeable shift in the cumulative curve
on day 2, accompanied by a marked elevation in cell number
per fiber, was seen (Fig. 3A, right panel), indicating the
LELI fibers survive. This is in agreement with previous conditions cells die by apoptosis whereas those around remain quiescent. Another possibility is that under these serum-starvation conditions, control fiber-associated cells

Effects of LELI on cell survival
The results shown in Fig. 2 raised the possibility that under serum-starvation conditions, control fiber-associated cells remain quiescent. Another possibility is that under these conditions cells die by apoptosis whereas those around LELI fibers survive. This is in agreement with previous demonstrations of the apoptotic effect of serum starvation in other cell systems (Kulkarni and McCulloch, 1994; Hasan et al., 1999) as well as in myoblasts (Chinni et al., 1999; Conejo and Lorenzo, 2001). To further investigate the protective effect of LELI under conditions of growth-factor deprivation, we tested the expression of BAX, a death-promoting molecule, and Bcl-2, a survival protein, in single fibers that were non-irradiated or had undergone LELI under serum-free conditions. Two fibers were plated in each 35 mm dish as far apart as possible, and only one half of the dish (with one of the fibers in it) was irradiated, and the other half served as a control. One day post irradiation, both fibers were fixed and reacted with antibodies against either Bcl-2 or BAX. In each of the three replications of this experiment, high levels of BAX were seen in the control fibers compared with zero levels in the irradiated fibers (Fig. 4A,C). Conversely, high levels of Bcl-2 were seen in the irradiated fiber, in contrast to minimal levels of expression in the control fiber (Fig. 4B,D).

The upregulation of BAX in fibers kept under serum-free conditions suggested that their associated cells undergo apoptosis in a p53-dependent manner: p53 and its downstream protein p21 are involved in growth arrest or apoptosis following DNA damage or cellular stress (El-Diery et al., 1993; Ko and Privas, 1996; Levine, 1997). Moreover, p53 has been shown to induce apoptosis by upregulating the BAX gene (Miyashita and Reed, 1995). To analyze the modulations in expression of these proteins as a consequence of LELI, we employed a myogenic cell culture that had been previously shown to be driven into the cell cycle and to proliferate in response to LELI (Ben-Dov et al., 1999; Shefer et al., 2001). Growing i28 cells were switched to serum-free DMEM for 36 hours, after which they were or were not irradiated for 3 seconds (Shefer et al., 2001). Cells were left in DMEM and harvested 24 and 48 hours post irradiation, and equal amounts of protein were electrophoresed and analyzed for p53, p21, BAX and Bcl-2. Densitometry analysis revealed that at zero time, cells expressed p53 and Bcl-2 proteins, whereas low or undetectable levels of BAX and p21, respectively, were seen (Fig. 4E,F). The relatively high expression of p53 at this time point was due to cells being in cell cycle arrest (El-Dairy et al., 1993). Still, these cells had not undergone apoptosis, because they were previously shown to be capable of re-entering the cell cycle upon LELI or serum stimulation (Shefer et al., 2001). p53, as well as BAX and p21 protein levels, were higher in the control non-irradiated cells 24 hours post-irradiation than in the
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LELI cells (Fig. 4E,F). Conversely, expression of Bcl-2 was further increased in LELI cells, while remaining undetectable in the control non-irradiated cells, just as with the LELI fibers (Fig. 4D). However, after 48 hours, the difference between the LELI and control cells with respect to protein expression was less pronounced, except for p21 expression, which remained lower in the LELI cells relative to the controls.

Cell viability was determined by MTT assay, which detects alterations in cellular redox activity in mitochondria. Cells were treated as described in Fig. 4, and the number of viable cells was measured after MTT addition 24 and 48 hours post irradiation. The overall number of viable cells declined in all serum-free cultures. However, the reduction was much more pronounced in the control non-irradiated cells (Fig. 5A), with a sharp decrease in cell viability between zero and 24 hours. After 48 hours, only 30% of the non-irradiated cells remained viable, in contrast to approximately 60% viability in the LELI cells.

In a parallel experiment, nuclei were cytochemically labeled with Hoechst stain, and apoptotic nuclei were scored by morphological criteria (Fig. 5B). The number of cells designated apoptotic by these criteria, 24 hours post irradiation, showed an approximately threefold increase in the non-LELI cultures, compared with only a 1.5-fold increase after LELI (Fig. 5C). This trend persisted for only the next 24 hours, reaching approximately 30% apoptotic cells in the LELI group and nearly 50% in the control group. The difference between the LELI and control cells became smaller after 48 hours, suggesting an evident, albeit transitory, effect of LELI on apoptosis. Repeated irradiation after 24 hours did not improve the state of the cells, and they underwent the same level of apoptosis as did cells that had been subjected to LELI only once (data not shown).

**Discussion**

Previous demonstrations of the stimulatory effect of LELI on cell proliferation have been reported in primary mass cultures of satellite cells and myogenic cell lines (Ben-Dov et al., 1999; Shefer et al., 2001). However, such cultures do not closely mimic the cell biology of myogenic cells within skeletal muscle, particularly with respect to the condition of myogenic cells in the cell cycle. Myoblasts in tissue culture, even under low nutrient and growth factor conditions, do not accurately reproduce the G0 status of the dormant satellite cell. Accordingly, we extended the application of LELI to...
isolated single fibers, comprising the contractile muscle fiber and its associated satellite cells. It was important for our purposes that these latter cells are in G0 at the time of isolation and during the entire preparation (Rosenblatt et al., 1995), thus giving the closest available approximation of their condition in vivo.

Using this model system, we show that LELI promotes accumulation of satellite cells near isolated single fibers grown under serum-free conditions and that the addition of even trace amounts of serum augments this effect synergistically. Such accumulation appears to reflect both promotion in mitosis of myogenic cells and, at least under serum-free conditions, their preservation from apoptosis by LELI. Moreover, LELI aids the survival of fibers under these pro-apoptotic conditions.

The effect of LELI on fiber-adjacent cell proliferation

In accordance with our previous observations in mass cell cultures (Ben-Dov et al., 1999; Shefer et al., 2001), we found that LELI enhanced the activation and cell cycle progression of quiescent satellite cells on freshly isolated muscle fibers (Fig. 1). At the time of irradiation, these cells are in contact with the muscle fiber and have not divided (data not shown). In fact, the number of cells that accumulated around non-irradiated fibers over the entire time period (Fig. 2) was about equal to the number of satellite cells residing on the average EDL muscle fiber (Beauchamp et al., 2000), and therefore, proliferation need not have been involved (Fig. 1C). In contrast, proliferation has to have occurred to account for the 20 or more cells that accumulated around the LELI fibers during the 2 days after irradiation. Thus, LELI drives quiescent satellite cells into at least one round of proliferation under the low-serum conditions in which they do not otherwise show any such response. However, this cell accumulation near the irradiated fibers was limited and transient: under serum-free conditions, no further net increase in cell number occurred after the second day, and by day 5 a slight reduction was noted. Interestingly, a more rapid reduction in cell number, with clear signs of apoptosis, was evident in the mass cell cultures (Fig. 4B, Fig. 5). In previous studies of mass cultures under low-growth conditions, LELI stimulated cells into the cell cycle up to 24 hours post irradiation (Shefer et al., 2001) but beyond 48 hours, most cells did not incorporate BrdU (data not shown). The relatively prolonged effect of LELI on the proliferation of cells associated with isolated fibers might be attributed to factors secreted by the fibers in response to irradiation. However, additional LELI 24 hours after the initial exposure did not alter the course of events in either cell culture or fibers (data not shown), suggesting, as an alternative explanation, that factors other than those secreted from the fibers per se are required to preserve the initial survival effect of LELI. Indeed, a synergistic effect of LELI and serum on cell proliferation was observed in cultures that were kept at serum concentrations as low as 0.1% (Fig. 3B). Notably, even under those conditions, the fact that the cells were uniformly stimulated into one or two extra divisions implies that the proliferative effect of LELI involves only a transient release from the tightly controlled mechanisms of cell cycle regulation.
LELI enhances Bcl-2 and decreases BAX expression

Our demonstration that proliferation of satellite cells on myofibers is stimulated by LELI confirms and extends previous observations on mass cell cultures (Ben-Dov et al., 1999; Shefer et al., 2001). However, a novel finding in the present study was the enhanced survival of both the isolated fibers and fiber-associated myogenic cells as a consequence of LELI. This was clearly seen under serum-free conditions, where the surviving cells and the myofibers in LELI cultures contained demonstrably higher levels of the anti-apoptotic protein Bcl-2 and lower amounts of the pro-apoptotic protein BAX relative to control fibers. This effect was also seen in cultures that contained one irradiated and one non-irradiated fiber in the same culture well, suggesting that this effect was direct and not mediated by diffusible factors. Similarly, LELI diminished the apoptotic rate of serum-starved bulk cultures of myogenic cells, again accompanied by low levels of BAX and high levels of Bcl-2 proteins.

Our observations fit with the widely accepted view that BAX overexpression promotes cell death in response to apoptotic stimuli, whereas Bcl-2 inhibits it (Sentman et al., 1991; Strasser et al., 1991; Oltvai et al., 1993). BAX and Bcl-2 can form heterodimers, and overexpression of one antagonizes the effect of the other (Oltvai et al., 1993). Our finding of a clear reciprocal pattern of Bcl-2 and BAX expression in control versus LELI fibers and cultured cells strongly implicates regulation of these factors as part of the protective role of LELI against apoptosis. In this same vein, we have recently reported that LELI specifically activates the MAPK/ERK signaling pathway but not the SAPK/JNK signaling pathway (Shefer et al., 2001). The former is a key pathway promoting cell survival in response to growth factors (Xia et al., 1995; Gardner and Johnson, 1996; Shimamura et al., 1999), whereas targets for the latter (i.e. c-Jun, BAX) have been shown to play a role in apoptosis after growth-factor deprivation (Bossy-Wetzel et al., 1997; Miller et al., 1997). This explanation is also in line with our finding of lower levels of p53, p21 and BAX in LELI versus untreated cells 24 hours after irradiation, because it has been shown that activated p53 mediates growth arrest and apoptosis by activating the expression of a number of cellular genes, such as p21 and BAX (El-Diery et al., 1993; Miyashita et al., 1994). This inhibition may involve activation of the MAPK/ERK signaling pathway by LELI (Shefer et al., 2001), as this pathway has been shown to protect against p53-dependent apoptosis (Anderson and Tolkovsky, 1999).

Thus, it is possible that LELI overcomes p53-dependent apoptosis by inhibiting the increase in p53 induced by cellular stresses, such as growth-factor deprivation (Blandino et al., 1995; Hasan et al., 1999; Honda et al., 2000), with subsequent induction of its downstream genes. The protective effect of Bcl-2 upregulation in response to LELI in both fibers and cell cultures could also be mediated by the suppression of p53 expression (Miyashita et al., 1994). At the same time, despite the correlation between LELI-mediated cell survival and p53 regulation, we cannot rule out other mechanisms by which LELI may prevent apoptosis. For instance, LELI has been shown to elevate ATP content and preserve mitochondrial structure in infarcted heart (Oron et al., 2001) and liver (Passarella et al., 1984; Yu et al., 1997) of rats. Continuous ATP synthesis is necessary to maintain a constant H+ efflux and membrane potential, which is deregulated following the death signal (Vander Heiden et al., 1997) (reviewed in Gross et al., 1999). Interestingly, Bcl-2 has been reported to prevent mitochondrial dysfunction by regulating proton flux (Shimizu et al., 1998). A recent report of direct upregulation of Bcl-2 by IL-7 in immature thymocytes (Von Freeden-Jeffry et al., 1997) also leaves open the possibility that LELI directly induces upregulation of Bcl-2 at the post-transcriptional level.

Implications of LELI in muscle repair

Our current study on single fibers and our previous studies in vivo and in cell cultures (Weiss and Oron, 1992; Ben-Dov et al., 1999) suggest that LELI potentiates the entrance of quiescent satellite cells into the cell cycle, acting synergistically with serum elements to enhance their proliferation and survival. Moreover, the fact that neither
LELI’s enhancement of proliferation nor its protective effects against apoptosis were maintained for a long period argues against any mechanism involving cell transformation. These findings are of the utmost importance in attempts to improve muscle regeneration following injury.

Immediately after muscle injury (e.g., partial excision), the traumatized area also suffers ischemic injury and a lack of nutrients and oxygen supply. The latter is renewed as new blood vessels form in the injured area. Nevertheless, during the initial phase, apoptosis may take place in the injured fibers and satellite cells (U.O., unpublished). Apoptosis of myonuclei has also been shown in skeletal muscle during hindlimb unloading-induced atrophy (Allen et al., 1997). Results of the present study suggest that LELI could reduce this process. Indeed, the above-mentioned phenomenon was demonstrated in single fibers as well as in cultured cells in low-serum and serum-free media, which may mimic the initial, post-traumatic phase. Moreover, we have reported a profound cardio-protective effect of LELI for cardiomyocytes in the injured heart (Oron et al., 2001), further suggesting that LELI enhances cell survival. Taken together, we believe that LELI, as a relatively non-invasive technique that enhances both cell survival and proliferation, might provide an effective means of ameliorating the long-term consequences of muscle injury.

i28 cells were a generous gift from A. Wernig and A. Irintchev (University of Bonn, Bonn, Germany). The work was supported by the European Community (EC) Biomed grant (BMIH4-97-2767). G. Shefer was supported by fellowships from the Federation of European Biochemical Societies and European Molecular Biology Organization. L. Heslop is supported by Biotechnology BIO4 CT 95-0284 and the MRC Clinical Sciences Centre and J.G. Gross by the MRC Clinical Sciences Centre.

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