Primary Myogenic Cells See the Light: Improved Survival of Transplanted Myogenic Cells Following Low Energy Laser Irradiation

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Background and Objectives: There is a substantial need for finding new avenues to promote muscle recovery when acute skeletal muscle loss extends beyond the natural capacity of the muscle to recover. Maintenance and regeneration of skeletal muscles depend mainly on resident stem cells known as satellite cells. Nevertheless, there are situations in which a significant loss of muscle tissue exhausts the satellite cell pool. For such cases, cell therapy and tissue engineering are becoming promising alternatives. Thus far, attempts to supplement damaged host muscles with donor satellite cells by means of myoblast transplantation therapy were mostly unsuccessful due to massive and rapid loss of donor cells within few hours after transplantation. This study aims at following the effects of low-energy-laser irradiation on the fate of implanted myoblasts.

Study Design: Primary myogenic cells, harvested from male rat skeletal muscles, were irradiated with low energy laser, seeded on a biodegradable scaffold and expanded in vitro. The scaffold containing cells was transplanted into partially excised muscles of host female rats. Donor cells were identified in the host muscle tissue, using Y-chromosome in situ hybridization.

Results: In this study, we show that laser irradiated donor primary myogenic cells not only survive, but also fuse with host myoblasts to form a host–donor syncytium.

Conclusions: Our data show that the use of low energy laser irradiation (LELI), a non-surgical tool, is a promising means to enhance both the survival and functionality of transplanted primary myogenic cells. Lasers Surg. Med. 40:38–45, 2008.

Key words: myoblast; scaffolds; skeletal muscle; stem cell; survival

INTRODUCTION

Adult muscle tissues consist of terminally differentiated multi-nucleated myofibers, which have lost their ability to proliferate. Indeed, maintenance and regeneration of skeletal muscles depend mainly on a small population of muscle stem cells named satellite cells [1]. The adult muscle is a highly stable tissue with a weekly turnover of 1–2% in adult rodents [2,3]. Yet, upon injury, the adult muscle tissue demonstrates a remarkable regeneration capacity [4,5] supported by satellite cells that are stimulated to proliferate and fuse with surrounding myoblasts or pre-existing myofibers to regenerate their functionality [6]. In clinical situations such as degenerative diseases, acute injury and aggressive tumor ablation, the damage to the muscle tissue is beyond it’s natural capacity for regeneration [7]. For such cases, cell therapy and tissue engineering are promising alternatives. Expanded populations of satellite cells are the most appealing source for stem cell based therapies due to their tendency to undergo myogenic differentiation, proliferative properties, and ability to incorporate into post-natal skeletal muscle [8,9]. Tissue engineering typically includes seeding three-dimensional scaffolds with donor cells followed by implantation to patients in order to replace or support regeneration of damaged tissue. Three-dimensional tissue-culture implants involve in vitro seeding of autologous or allogenic cells onto the scaffold where cells proliferate, migrate, and differentiate while secreting extracellular matrix components required for constructing the tissue. Such scaffolds mimic the natural dynamics of tissue regeneration and are generally accepted to be superior to cell suspension [10,11]. In classical (congenic) myoblast transplantation therapy, massive and rapid loss of most donor myoblasts was shown to occur within the first few hours after transplantation. When satellite cells are enzymatically dissociated from their native environment, expanded and transplanted into a new host, their subsequent ability to

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contribute to muscle regeneration is inefficient to support muscle regeneration, even when using very large cell populations [12]. Among the mechanisms suggested to account for the loss of transplanted myoblasts are host inflammatory reaction responses, necrotic, and apoptotic processes [13–16]. Massive apoptosis of donor cells was also reported as a major problem with transplanted cardiomyocytes [17]. Therefore, finding a treatment aimed at improving the survival of myoblasts after transplantation is of major concern.

We had previously shown that low energy laser irradiation (LELI) of serum-starved satellite cell cultures or isolated muscle fibers reduces their apoptosis, possibly indicating the ability of LELI to promote cell survival [40]. We also found that LELI promoted the activation and proliferation of satellite cells in culture and in isolated muscle fibers [28,29], induced synthesis of cell-cycle regulatory proteins in tissue cultures of satellite cells, and activated early cell-cycle-regulatory genes [28]. These processes may play a part in the LELI-induced accelerated wound healing and skeletal muscle regeneration after injury [18–20]. LELI was also shown to modulated various biological processes such as increasing mitochondrial respiration and ATP synthesis [21–24], and promoting the proliferation and maturation of human osteoblasts in vitro [25]. Furthermore, others and us demonstrated that LELI does not have any deleterious consequences in culture and in vivo [26,27]. Based on the beneficial impact of LELI on primary myoblast activation and survival, we aimed here at following the effects of LELI on the fate of myoblasts 1 and 2 weeks after transplantation. We hypothesized that irradiation of donor satellite cells and the site of implantation may enhance myoblast proliferation, reduce apoptotic processes and thereby promote the survival of donor satellite cells and the efficiency of regeneration.

MATERIALS AND METHODS

Animals

Twenty-four Charles River male rats at the age of 3 weeks (50–70 g) served as donors and 24 Charles River female rats at the age of 3 months (200–250 g) were used as hosts. Rats were housed under a 12:12-hour light-dark cycle in a temperature controlled room. Food and water were provided ad libitum. Experimental procedures were conducted according to the Institutional Animal Care and Use Committee of the Tel-Aviv University.

Low Energy Laser

He–Ne laser (632.8 nm, 4.5 mW; 1.8 mm beam diameter; Ealing Electro-Optics, Holiston, MA) was used to irradiate primary satellite cells and the site of transplantation. Power density of 200 mw/cm² and energy density of 0.6 J/cm² were applied to the cells.

Isolation of Satellite Cells and Immunohistochemistry

Rat muscle satellite cells were prepared as previously described [28]. Briefly, the Gastrocnemius, Gluteus, and Soleus muscles were excised from male rats. The muscles were trimmed of fat and connective tissue, and chopped with scissors. The tissue was digested for 1 hour at 37°C with 1.25 mg/ml Pronase (Boehringer, Mannheim, Germany), and then with 0.25% w/v trypsin for 30 minutes at 37°C. Tissue fragments were collected by centrifugation at 1,500g for 5 minutes and then titrated through a fine pipette. Differential centrifugations were used to enrich the cell fraction [28]. The final cell-pellet was re-suspended in DMEM containing 1% antibiotic solution and 10% horse serum (HS), passed through a 200 μm nylon membrane and collected to a 15 ml conical tube. The total collected volume was divided to 3 ml per 60 mm uncoated cell-culture dishes. These dishes were incubated for a period of 2 hours (pre-plating) after which, half of the dishes served as non-irradiated cell controls and the other half were laser irradiated for 3 second periods using the dot grid method as described previously [28,29]. Briefly, dishes containing non-irradiated (control) cells were kept outside the incubator alongside the irradiated cultures during the entire irradiation period. A transparent glass (200 mm×100 mm) with a grid of adjacent circles (marked at minimal spacing so that centers of each two adjacent circles were 1.8 mm apart) was used to uniformly irradiate the entire tissue-culture dish. The grid glass was supported horizontally on 40-mm high aluminum legs, forming a “miniature grid table” on top of which a 60 mm tissue culture dish was placed for laser irradiation. The entire table with the grid and dish could be then smoothly shifted form one grid dot to the next without agitating the cells. A metronome was utilized for providing 3-second intervals between switching from one irradiated dot on the grid to the next. After irradiation, only the non-adherent primary satellite cells were collected from irradiated and non-irradiated dishes. These cells were counted, using a hemocytometer, and the total number of cells was determined. Cells (3×10⁵) were either plated on 0.1% gelatin-coated 60 mm tissue culture dishes for determining lineage identity or were seeded on gel matrix for in vitro expansion prior to transplantation as described below. Cells were supplemented with 10% HS in DMEM (growth medium) and were further incubated for 3 days. To determine the myogeneity of cultures at the day of transplantation, cells grown in culture dishes were fixed and immunostained with a muscle specific transcription factor antibody, MyoD mouse mAb (IgG1, clone 5.8A, BD Biosciences, San Jose, CA; 1:500, as previously described [30].

Matrix Preparation and Seeding of Primary Cells

Sterile gelatin flakes (0.09 g; Gelfoam, Upjohn-Pharmacia, Kalamazoo, MI) were dissolved in 35 ml of growth medium, gently mixed and incubated at 37°C for 3 hours to create a nutrient saturated gel matrix. Half milliliter of gel matrix was transferred to a 5 ml sterile tube, and was further diluted with 0.5 ml of growth medium. Twenty-four such tubes were prepared. A total of 3×10⁵ cells were added to each gel-matrix containing tube immediately after the isolation and irradiation.
procedure. Tubes were gently agitated to facilitate dispersal of cells into matrix. Cells were expanded in vitro for 3 days prior to the transplantation, based on our preliminary studies showing that the population more than triples within this time frame. To refresh the growth medium—tubes were centrifuged at 1,500g for 5 minutes, condensing gel-matrix volume by half. Medium was collected and decanted into a new tissue culture dish; fresh growth medium was added to each tube that was then gently agitated until gel matrix regained its volume. Trypan blue was added to the collected growth medium for visualization of both live and dead cells. Only negligible numbers of cells were detected in the old medium.

**Partial Excision and Cell Implantation**

Female rats (n = 24) were anesthetized using Avertin (1 ml of 1.25% tribromoethanol in saline per 100 g body weight). The left hind leg was shaved, disinfected with 70% ethanol and the tibialis anterior (TA) muscle was exposed. Using a device made of two surgical blades fixed at a constant distance and a constant depth, two parallel cuts were made. Muscle tissue between those cuts was excised, removed, weighed, and skin was surgically stapled. The average weight of the excised portion of TA muscle was 199.5 mg, which typically comprise 0.24% of the muscles’ weight. Half ml of durabiotic antibiotics (30 U/100 g, Teva, Israel) was injected intra musculary to the thigh after the surgery. Rats were returned to their cages in the animal facility for additional 2 days, allowing inflammation processes to decrease to avoid interference of the major inflammatory response with the transplantation procedure.

Two days after partial excision procedure animals were anesthetized as described above. The partially excised left hind leg was shaved and disinfected with 70% ethanol. The precise place of TA injury was re-traced and 100 μl of matrix containing one million of either LEL-irradiated or non-irradiated cells were inserted into the muscle using a sterile spatula. After the implantation of LEL-irradiated cells, the site was LEL-irradiated at two tangential consecutive sites for 2 minutes each (n = 12 rats; Laser group). When non-irradiated cells were transplanted, no in vivo irradiation was done (n = 12 rats; Control group). Skin was sutured and 2 ml saline and 0.8 ml durabiotics were administered IP and IM, respectively. Rats were kept in a warmed cage for 5 hours after which they were returned to the animal facility.

**Histological Procedure**

Animals were sacrificed 1 (n = 12) and 2 (n = 12) weeks after transplantation. TA muscles were dissected out, and the site of the injury was identified and separated from the muscle. Muscle samples were fixed overnight in fresh 4% paraformaldehyde solution, embedded in paraffin, and deparaffinized as described previously [31]. Each paraffin block was cut to 5 μm thick longitudinal serial sections, which were collected on positively charged microscope slides (OptiPlus, BioGenex, San Ramon, CA). Every alternate successive section was hybridized with the Y-chromosome-specific DNA probe and immunoreacted with proliferating cell nuclear antigen (PCNA).

**In Situ Hybridization for DNA**

To identify male donor cell nuclei present in the female hosts, in situ hybridization with a Y-chromosome-specific DNA probe was performed as previously described [32].

**Proliferating Cell Nuclear-Antigen Immunostaining**

Muscle sections were deparaffinized and immunostained using an antibody against PCNA (a marker for dividing cells), using a commercial kit from Zymed laboratories (Invitrogen, Carlsbad, CA). The cells were then counter-stained with haematoxylin as previously described [33]. Control slides that were reacted with blocking solution without primary antibodies were processed in parallel.

**Light Microscopy**

Observations were made with an Olympus light microscope and the images were acquired with an Olympus Camera.

**Scanning Electron Microscopy (SEM)**

Samples of gel matrix were analyzed 3 days after seeding with myogenic cells. Samples were dehydrated in ethanol serial steps, and coated with gold in a sputtering device for 3 minutes at 15 mA. Specimens were examined by SEM (JEOL-840).

**Count of Cells Seeded in Gel Matrix**

Samples of gel matrix seeded with cells were collected daily, smeared on a glass slide, and fixed with methanol. Samples were stained with Giemsa and the total number of nuclei from six arbitrary fields per sample were counted (n = 3 samples per time point). To calculate growth curves we grew cultures of gel-matrix for 10 days. Cell counts revealed a logarithmic growth curve during the first week; however, cell number diminished from day 8 and forth.

**RESULTS**

Myogenic primary cells were harvested, irradiated with low energy He–Ne laser and expanded on a gel-based matrix for 2 days to create a scaffold supported 3D culture. The expression of the muscle specific transcription factor MyoD was analyzed to confirm the myogeneity of primary cultures (Fig. 1). Light and electron microscopy images of the gel matrix seeded with primary myogenic cells show that cells are attached to the partially dissolved component of the gelatin flakes within the three-dimensional matrix (Fig. 2). Both irradiated and non-irradiated cells grown on a gel matrix showed a logarithmic growth curve (data not shown).

**One Week Post-Transplantation**

The histology of the injured site of both laser and control rats was characterized by unorganized amorphous tissue with obvious intrusions of gel-matrix surrounded by well-organized myofibers containing myonuclei of host origin and a dense population of proliferating cells (PCNA+; Fig. 3).
Fig. 1. Expression of the muscles specific transcription factors MyoD (a: red and c: green) in a 3 and 10-day-old primary myogenic cultures, respectively. Cultures were stained with DAPI (b, d: blue) to visualize total nuclei. Note that 18 out of 21 cells in a 3-day-old primary culture (a, b) express MyoD in their nucleus, and are therefore referred to as myogenic. Differentiated cells and contractile myofibers are depicted in the phase contrast image of a representative 10-day-old irradiated primary culture (e). Scale bar, 20 mm. [Figure can be viewed in color online via www.interscience.wiley.com.]

Fig. 2. In vitro images from scanning electron (a, b) and light (c, d) microscope of the gel-matrix, seeded with primary myogenic cells and stained with Giemsa. Arrow points to a myogenic cell nucleus. [Figure can be viewed in color online via www.interscience.wiley.com.]
While cells of donor origin, shown by nuclei positive for Y-chromosome, are readily found in the laser rats (Fig. 4a) donor cells in the control rats were not detected (Fig. 4c,d).

**Two Weeks Post-Transplantation**

At 2 weeks after the transplantation of the LEL-irradiate cells, Y-chromosome-positive donor nuclei were detected within host myofibers and young myotubes (the latter are recognized by their central nuclei) in conjunction with host nuclei (termed “hybrid myotube”; Fig. 5). Notably, in muscles transplanted with non-LELI donor cells no Y-positive nuclei were detected in any of the hosts, 2 weeks after transplantation. While unorganized tissue was seen in the transplantation site after 1 week, 2 week after transplantation the cells were aligned along the longitudinal axes of the muscle as defined according to the direction of intact host myofibers (Fig. 5). In addition, the biodegradable gel-matrix could no longer be seen at the site of the regenerating tissue of both laser and control rats.

**DISCUSSION**

Myoblast transfer of normal or genetically modified cells is a promising route for clinical treatment of muscle pathologies. Isolation of primary myogenic cells from muscle biopsies and their in vitro expansion are relatively straightforward procedures. The main holdback, however, is that most myoblasts grafted into the skeletal muscle or myocardium are eliminated within the first 2 days after transplantation. Acute inflammation was suggested to play an important role in this donor cell death [13,16,34–36]. In the present study, we showed that LEL-irradiated, expanded, and transplanted primary myogenic donor cells, survive and fuse with host myogenic cells to form host–donor syncytia. Male donor cells were transplanted into female host rats 48 hours after their TA muscles were partially excised, thus bypassing the most potentially destructive stages of the inflammation process [37–39]. We used Y-chromosome as a natural marker to track male donor myoblasts after implantation by means of in situ hybridization. This “intrinsic marker” approach is advantageous over marker-gene transfections or radioactive labeling of the donor cells, which may interfere with natural processes taking place after transplantation. For cell transplantation we used a 3D scaffold as these scaffolds are considered a superior method compared with mere injection of cell suspension in transplantation procedures (for more details, see Reference [10]).

Our results indicate that 1 week post-transplantation, the morphology of the injured area of LEL-irradiated TA muscles was characterized by unorganized and amorphous tissue comprised of a dense population of proliferating host and donor cells with apparent remnants of the gel-matrix. Two weeks after transplantation, however, cells in the injured area were aligned along the longitudinal axes of the

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Fig. 3. Representative longitudinal sections of the TA muscles 1 week after transplantation with irradiated (a,b) and non-irradiated (c) primary myogenic cells. A dense population of proliferating cells (PCNA+ cells, deep purple, black arrows) is depicted in high (a,c) and low (b) magnifications of TA muscle. Intrusions of the gel-matrix (green arrows) can be clearly observed. PCNA positive cells are observed between intact myofibers (yellow arrows) within the injured area. Scale bars, 20 μm (a) and 100 μm (b). [Figure can be viewed in color online via www.interscience.wiley.com.]
muscle. LEL-irradiated donor cells remained abundant after 2 weeks from transplantation, whereas no Y-chromosome positive cells were detected in any of the host muscles. The latter result accords with previous studies in which extensive death of transplanted donor myoblasts as early as 2 days after transplantation is reported [13,16,34]. Our results show that LEL-irradiated donor cells survived, fused with host myofibers and were found in conjunction with host nuclei in hybrid myotubes (syncytia). The gel-flake based matrix seen as isolated islands in the tissue 1 week after transplantation, was no longer detected in the tissue a week later, demonstrating a rapid and complete degradation without compromising tissue integrity.

A possible explanation for the enhanced survival of the transplanted LEL-irradiated myogenic cells may lie in the protective effect of LELI against apoptosis. Such a

![Fig. 4. Representative longitudinal sections of the TA muscle implanted with non-irradiated (a,b) or irradiated (c,d) primary myogenic cells, 1 week post-transplantation at low (a,c) and high (b,d) magnifications. Green arrows point to Y-chromosome positive donor nuclei, mainly found in unorganized host tissue (c,d). Black arrows point to host nuclei found within host myofibers (a–d). Scale bar, 20 μm. [Figure can be viewed in color online via www.interscience.wiley.com.]

![Fig. 5. Representative longitudinal sections (a,b,c) of TA muscles from three rats, 2 weeks after transplantation with LEL-irradiated donor myogenic cells. Donor cells (green arrows) survive and their nuclei (stained brown) are found both in host myofibers and in myotubes in conjunction with nuclei of host origin (stained blue, black arrow) termed “hybrid myotube.” Scale bar, 20 mm. [Figure can be viewed in color online via www.interscience.wiley.com.]
protective effect was shown to take place in both cultured satellite cells and isolated myofibers, held in serum-free conditions that normally induce apoptosis [40]. In both systems, expression of the anti-apoptotic protein, Bcl-2, was markedly increased whereas expression of the pro-apoptotic protein BAX was reduced compared to the non-irradiated control group. In culture, these changes were accompanied by reduced expression levels of p53 and the cyclin-dependent kinase inhibitor p21, implicating a p53-dependent anti-apoptotic effect of LELI on the irradiated cells [40]. Nevertheless, it may well be that LELI inhibits apoptosis via additional pathways. Massive apoptosis of donor cells was also reported as a major problem in cardiomyocytes transplantation to ischemic heart. However, exposure of cultured cardiomyocytes to heat shock before transplantation strikingly increased their survival at the first day post-transplantation [17]. Similarly, LELI-irradiation of acute ischemic dog and rat hearts was shown to promote the survival of cardiomyocytes by increasing the content of heat shock proteins of the antioxidative enzyme catalase and by better preserving their mitochondria [41]. We additionally showed that LELI transduces its signal specifically via the MAPK/ERK pathway rather than stress signaling pathways such as JNK or p38 MAPK [40]. In all, it seems that LELI contributes to enhanced survival of cells via stress dependent and independent mechanisms. The contribution of transplanted satellite cells to host muscle regeneration is usually inefficient, even when 10^5–10^6 donor cells were engrafted in a host muscle [12]. Taking a different approach of using freshly isolated myofibers grafted into muscles of dystrophic mdx-nude mice revealed that the a few satellite cells associated with one myofiber can give rise to sufficient differentiation-competent progeny capable of generating thousands of myonuclei [42]. The apparent drawback of this latter approach lies in the high-apparent drawback of this latter approach lies in the high-
skill procedure involving the isolation of individual intact systems, expression of the anti-apoptotic protein, Bcl-2, and by better preserving their mitochondria [41]. We additionally showed that LELI transduces its signal specifically via the MAPK/ERK pathway rather than stress signaling pathways such as JNK or p38 MAPK [40]. In all, it seems that LELI contributes to enhanced survival of cells via stress dependent and independent mechanisms. The contribution of transplanted satellite cells to host muscle regeneration is usually inefficient, even when 10^5–10^6 donor cells were engrafted in a host muscle [12]. Taking a different approach of using freshly isolated myofibers grafted into muscles of dystrophic mdx-nude mice revealed that the a few satellite cells associated with one myofiber can give rise to sufficient differentiation-competent progeny capable of generating thousands of myonuclei [42]. The apparent drawback of this latter approach lies in the high-skill procedure involving the isolation of individual intact myofibers, holding it from being reproduced in industrial type bioreactors. The study presented here shows that the relatively straightforward procedure of LELI has the potential to increase the survival of transplanted progenitor muscle cells, promote their fusion with the host myofibers and enhance their ability to recover. An essential advantage held by this method is therefore that it offers highly reproducible and simple procedure with the beneficial effect of enhancing the efficiency of cell-transfer therapy.

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