Published in final edited form as:

Biomaterials. 2011 December ; 32(34): 8905–8914. doi:10.1016/j.biomaterials.2011.08.019.

THE ROLE OF MULTIFUNCTIONAL DELIVERY SCAFFOLD IN THE ABILITY OF CULTURED MYOBLASTS TO PROMOTE MUSCLE REGENERATION

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Abstract

Many cell types of therapeutic interest, including myoblasts, exhibit reduced engraftment if cultured prior to transplantation. This study investigated whether polymeric scaffolds that direct cultured myoblasts to migrate outwards and repopulate the host damaged tissue, in concert with release of angiogenic factors designed to enhance revascularization of the regenerating tissue, would enhance the efficacy of this cell therapy and lead to functional muscle regeneration. This was investigated in the context of a severe injury to skeletal muscle tissue involving both myotoxin-mediated direct damage and induction of regional ischemia. Local and sustained release of VEGF and IGF-1 from macroporous scaffolds used to transplant and disperse cultured myogenic cells significantly enhanced their engraftment, limited fibrosis, and accelerated the regenerative process. This resulted in increased muscle mass and, improved contractile function. These results demonstrate the importance of finely controlling the microenvironment of transplanted cells in the treatment of severe muscle damage.

INTRODUCTION

Musculoskeletal diseases present a significant burden to patients and health care systems [1,2]. In normal/healthy muscle, highly specialized myofibers, the basic contractile units of skeletal muscle, have an intrinsic ability to contract and generate movement. In injured muscles, the loss of myofiber contractility can induce severe functional deficiencies.

Although a variety of cell populations have been implicated in muscle regeneration, including muscle-resident side population cells (muSP) [3], multipotent adult progenitor cells (MAPC) [4–6], and bone marrow-derived cells [7–10], the activation of the satellite cells, a quiescent specialized sub-population of adult stem cells localized within the basal lamina of the myofibers, is believed to be primarily responsible for physiologic muscle-regeneration. Primary myoblasts derived from satellite cells readily proliferate in culture, providing in many ways an ideal cell source for transplantation therapies when host cells are insufficient or incapable of robust regeneration. However, cultured murine myoblasts demonstrate reduced engraftment efficiency when subsequently transplanted [11,12]. In contrast, direct transplantation of murine muscle stem cells derived from the satellite cell

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population, with no culture period, can lead to efficient engraftment and muscle regeneration [11–13].

Transplant strategies that improve cultured myoblasts ability to engraft and promote functional regeneration could provide a major advance in muscle regeneration, and will likely be required to treat humans due to the large number of repair cells in clinical settings. As a comparison, the mouse tibialis anterior (TA) muscle has a volume of 30 mm³ [14] while the human TA is 130,000 mm³ [15]. The regenerative index for subpopulations of non-cultured murine skeletal muscle precursor cells has been reported to lie between 2,000 and 4,000 (number of donor-engrafted murine muscle fibers generated per 10⁵ transplanted cells) [11,12], a number quite close to the total number of muscle fibers in the mouse TA (4100) [16]. If human cells have similar regenerative capacity, approximately 100–200 million precursor cells would be required to repair a damaged adult human TA. On average, a 100 mg vastus lateralis human muscle biopsy yields 5,000 myogenic clones [17], so 2–4 kg of muscle would be required to yield the appropriate number of non-cultured skeletal muscle precursor cells to repair the human TA, assuming all of these myogenic clones fit into the subpopulation of precursor cells. As it is impractical to obtain 2–4 kg of muscle tissue from a patient to promote regeneration, this simple analysis suggests it will be necessary to culture expand cells in order to promote muscle tissue regeneration in humans. This motivates the development of strategies that can enhance the capability of these cells to promote regeneration.

Myoblast fate in vivo is finely regulated by a number of microenvironmental signals, including both extracellular matrix molecules (ECM) and soluble signaling molecules [18]. It is possible to mimic various aspects of the ECM with synthetic matrices that contain covalently coupled peptides that replicate key properties of the ECM. The finding that fibronectin is especially relevant in the early proliferative phase of myogenesis has led to the covalent modification of alginate with the adhesion oligopeptide G₄RGDSP to enhance transplanted myoblast survival and proliferation [19]. A variety of trophic factors also control the proliferation and differentiation of myogenic cells, including both inflammatory cytokines [20], and growth factors [21] (e.g., insulin growth factors [22,23], and these play key modulatory roles in muscle growth and regeneration. It was previously reported that the release of single or multiple growth factors (e.g. HGF, FGF-2, VEGF, IGF-1, PDGF-BB) [21,23,24] from natural or synthetic matrices [23–25,27] can be finely tuned to allow well controlled signaling. In particular, the dual delivery of angiogenic (VEGF) and myogenic (IGF-1) factors from a biodegradable injectable alginate hydrogel has been specifically demonstrated to enhance functional muscle regeneration following hindlimb ischemia [23].

This study addressed the hypothesis that one can promote functional skeletal muscle regeneration in a severely injured muscle by cultured myoblast transplantation with an appropriate carrier. In particular, a macroporous RGD-containing peptide alginate scaffold was designed to enhance cell viability during transplantation, while mobilizing the cells to disperse and engraft through a large tissue volume following transplantation. The scaffold simultaneously provides a localized and sustained presentation of factors that modulate angiogenesis (VEGF) and myogenesis (IGF-1). The goal was to design a strategy (cell-instructive-scaffold) to prevent transplanted cells from undergoing apoptosis, and instead be activated and enter in the proliferative phase, migrate outward to the site of injury, fuse and differentiate in order to enhance repopulation of injured muscle and increase regeneration (Fig. 1). A severe murine model of muscle injury was used in these studies by combining myotoxin-induced direct skeletal muscle damage [28] with induction of severe ischemia to the hindlimb [23,25]; in contrast, our previous studies utilized muscle injury alone [21] or ischemia alone [23]. Donor myoblasts were isolated from transgenic mice constitutively expressing GFP in all cells to allow tracking in vivo, and were culture expanded prior to

transplantation. A variety of facets of muscle regeneration, including fiber regeneration, muscle mass, vascularization, and most importantly, muscle contractile function were analyzed.

MATERIALS AND METHODS

Alginate modification and scaffold fabrication

Ultrapure alginates were purchased from ProNova Biomedical (Norway). MVG alginate, a high-G-containing alginate (M/G ratio of 40/60 as specified by the manufacturer) was used as the high molecular weight (250,000Da) component to prepare gels. Low molecular weight (LMW) alginate (50,000Da) was obtained by γ -irradiating high molecular weight alginate, as specified by Kong et al. [41]. Both alginate polymers were diluted to 1% w/v in double-distilled H₂O, and 1% of the sugar residues in the polymer chains were oxidized with sodium periodate (Aldrich, St Louis, MO, USA), as previously described [41]. An equimolar amount of ethylene glycol (Fisher, Pittsburgh, PA, USA) was added to stop the reaction, and the solution was subsequently dialyzed (MWCO 1000, Spectra/Por[®]) sterilized by filtration, lyophilized and stored at -20°C. Both alginates were modified with covalently conjugated oligopeptides with a sequence of G₄RGDSP (Commonwealth Biotechnology, Richmond, VA) at an average density of 3.4 mM peptide/mole of alginate monomer using carbodiimide chemistry, as previously described [19]. To prepare gels, modified alginates were reconstituted in EBM-2 (Cambrex Corporation, Walkersville, MD, USA) to obtain a 2% w/v solution (50% LMW, 50% MVG used in all experiments) prior to gelation. The 2% w/v alginate solutions were cross-linked with aqueous slurries of a calcium sulphate solution (0.21 g CaSO₄/mL distilled H₂O) at a ratio of 25:1 (40 μ L of CaSO₄ per 1 mL of 2% w/v alginate solution) using a 1-mL syringe. Alginates were first mixed with recombinant human VEGF₁₆₅ protein (Biological Resources Branch of National Cancer Institute) and with recombinant human IGF-1 (R&D System) by using two syringes coupled by a syringe connector at a final concentration of 60 μ g/mL for each protein. The calcium slurry (Sigma, St Louis, MO, USA) was then mixed with the resulting alginate/growth factor/s solution using two syringes coupled by a syringe connector to facilitate the mixing process and prevent entrapment of air bubbles during mixing. The resulting solution was immediately placed into molds of 2 mm depth. A sterile glass plate was placed over the molds and, after the alginate had completely gelled (30 min), squares of 5 mm $\times$ 5 mm were cut using a punch. To produce macro-porous scaffolds with open interconnected pores, the gels were cooled to -80°C, and the gels were lyophilized/freeze dried and stored at -20°C until cell seeding. Fifty μ L (200,000 cells/gel) of a cell suspension (4 $\times$ 10⁶ cells/mL) was gently poured onto the modified, open-pore polymer scaffolds. The gel were incubated for about 20 min before adding 500 μ L of complete culture medium, then maintained at 4°C prior to animal implantation for ~20 min prior to implantation.

Myoblast purification, characterization and cultures

Primary myoblasts were derived from 4–12 weeks-old wild type C57BL/6 and transgenic Tg(ACTbEGFP)1Osb, constitutively expressing GFP in all cells. The cells were isolated from hindlimbs, as described [42]. Cells were collected via centrifugation and cultured until 80 % confluent (about 7 days) and subsequently purified via Percoll (Amersham Biosciences, Uppsala, Sweden) density gradient fractionation. The gradient consisted of 3 mL of 20 % Percoll diluted in PBS (Gibco), 3 mL of 30% Percoll diluted in DMEM (Invitrogen) and 35 % Percoll diluted in PBS (Gibco). Cells were immediately centrifuged at 1600 rpm for 20 min at 25°C. The cells from the 30% fraction were collected and resuspended in complete culture medium (high glucose DMEM with added pyruvate (Gibco) supplemented with 10% fetal bovine serum (FBS) and 10% penicillin/streptomycin (P/S, Gibco) and used for transplantation. To characterize myoblast cultures, Percoll purified

primary myoblasts were stained with desmin (1/100; Santa Cruz Biotechnology, Santa Cruz, CA) as described [42]. The percentage of myogenic cells was determined microscopically as the ratio of desmin expressing cells to the total number of cells in 10 randomly chosen fields. The resulting cultures consisted of a 95% desmin-positive population.

Animals and tissue injury

All animal work was performed in compliance with NIH and institutional guidelines. GFP transgenic mice (C57BL/6-Tg(ACTbEGFP)10sb) were used as a cell source, and six-seven weeks-old female wild type C57BL/6J mice (Jackson Laboratories, Bar Harbour, ME, USA) were used for treatments. Mice were anesthetized with an intraperitoneal injection of a mixture of ketamine 80 mg/kg and xylazine 5 mg/kg prior to all surgical procedures. For myotoxin injuries, the tibialis anterior muscles of the right legs of anesthetized mice were injected with 10 µl of 10 µg/ml Notexin Np myotoxin from Notechis Scutatus snake venom (Latexan) using a 5 µl Hamilton syringe. Six days after notexin injection, hindlimb ischemia was induced by unilateral external iliac and femoral artery and vein ligation, as previously described [29]. After the vessel ligation, the middle part of tibialis muscle was treated with a scaffold. The incision was surgically closed, and animals monitored over time.

Ischemia and perfusion analysis

Measurements of the ischemic/normal limb blood flow ratio were performed on anesthetized animals ($n=10$) using a LDPI analyzer (Perimed AB, Stockholm, Sweden) as previously described [42]. Perfusion measurements were obtained by scanning entire hindlimbs under basal conditions and then weekly after surgery, and the ratio of perfusion between ischemic to non-ischemic limb of the same animal was calculated.

Histological assessment of skeletal muscle

At 3 days, 2 and 6 weeks following induction of ischemic injury, anesthetized mice were sacrificed and hindlimb muscle tissues ($n=10$ /time point/condition) were processed for histological analyses. For regeneration metrics, the samples were stained with hematoxylin and eosin. Longitudinal section images were captured at 20X magnification and merged in Adobe Photoshop (Adobe systems, San Jose, CA) and then the number of centrally located nuclei was manually measured and tallied. The remaining defect areas were identified in longitudinal hematoxylin/eosin-stained sections of isolated tibialis anterior muscles via their lack of organized muscle fibers and presence of disorganized matrix. Analysis was performed by using Adobe Photoshop (Adobe Systems, San Jose, CA). Five samples were analyzed for each condition. Vascular ECs were identified by immunostaining for mouse CD31 (BD Biosciences Pharmingen, San Diego, CA, USA). All the merged tissue sections were randomly analyzed, as previously described [23]. Sections from each sample were visualized at 200 and 400 with an Olympus IX81 light microscope (Japan) connected to an Olympus DP70 digital image capture system (Japan), and analyzed using IPLab 3.7 software (Scanalytics, Rockville, MD, USA).

For slow and fast muscle fiber type staining, sections of tibialis muscle were stained separately for fast and slow muscle fiber types using MY32, a monoclonal mouse anti-fast myosin heavy chain (fMHC) antibody (1:400, Sigma) and NOQ7.5.4D, a monoclonal mouse anti-slow myosin heavy chain (sMHC) antibody (1:200; Sigma), and a secondary antibody solution containing biotinylated anti-mouse IgG (Vector) diluted 1:300 in PBS containing 1% BSA and 0.05% Tween for 2 hours at RT. Samples were mounted using ProLong® Gold Antifade Reagent with DAPI (Molecular Probes). Mouse diaphragm muscle cross-sections were also stained using this protocol to ensure specificity of the stains.

GFP expression was detected in both cryo-section and paraffin-embedded sections by direct GFP fluorescence and by anti-GFP immunofluorescence, respectively. In particular, muscle paraffin-sections were permeabilized with 1 % BSA-0.2% Triton X100/PBS and 5 % goat serum, and stained with 1:50 chicken anti-GFP Ab (Molecular Probes) and, 1:200, 488 goat Alexa Fluor anti chicken Ab (Molecular Probes). Interstitial fibrosis was morphometrically assessed in Masson Trichrome (Sigma Aldrich) stained sections.

Mechanical measurements

At 3 days, 2 and 6 weeks following the treatment, C57BJ6 mice ($n= 5$ /conditions) were anesthetized before muscle isolation and then sacrificed by cervical dislocation. Intact tibialis muscles for each condition (blank alginate, scaffold VEGF/IGF-1, scaffold cells and VEGF/IGF-1, bolus of cells VEGF₁₆₅/IGF-1) and the uninjured contralateral hindlimb were dissected for isolated muscle force measurements, as specified by Borselli et al. [23].

Statistical Analyses

All results are expressed as mean $\pm$ standard deviation (SD), unless otherwise noted. Multivariate repeated-measures ANOVA was performed to test for interactions between conditions. Differences between conditions were considered significant if $p < 0.05$.

RESULTS

Engraftment of transplanted myoblasts

The tibialis muscle of each recipient C57BJ6 mouse was subjected to intramuscular injection of notexin six days prior to cell therapy, as this time-frame allows the myotoxin-induced necrosis to diffuse throughout the muscle tissue [29]. Mice were subsequently subjected to unilateral external iliac and femoral artery ligation to induce severe hindlimb ischemia [23,25]. A complete loss of locomotion of the injured hindlimb was immediately observed in with this treatment. Analysis of tissue sections at early times following dual injury revealed a largely necrotic defect with diffusely disorganized and damaged myofibers resulting from this combined injury (Fig. 2a, b). This injury was more severe than that previously seen from ischemia injury alone [23], and led to a greater loss of the mechanical function of the tibialis.

Following vessel ligation, the middle part of the tibialis muscle was treated by placing a macroporous, degradable RGD-modified alginate scaffold delivering GFP-myoblasts and/or growth factors over the necrotic muscle. Four specific treatments were analyzed: (i) blank alginate, (ii) scaffold delivering VEGF (3 μ g) and IGF-1 (3 μ g), (iii) scaffold delivering VEGF/IGF-1 (3 μ g each) and GFP-satellite cells (200.000 cells/gel) and, (iv) bolus injection of GFP-satellite cells (200.000 cells) and VEGF/IGF-1 (3 μ g each) in PBS. A control of the scaffold delivering only myoblasts was not included, since a previous study in a less severe injury model demonstrated little effect with this condition [21]. In all experiments the uninjured hindlimb of the same animal (no injury nor treatment) was used as a control. All scaffolds were still localized at the initial implant site at the time of retrieval (3 days, 2 and 6 weeks; Fig. 2e).

The capacity of transplanted, GFP-positive cultured myoblasts to engraft into damaged muscle, and to act as a regenerative precursor population was first analysed. Analyses of tibialis muscles in recipient mice at 3 days post-treatment was performed using antibody staining to GFP, and confirmed a robust GFP myoblast engraftment into the host muscle when cells were transplanted on scaffolds releasing VEGF/IGF-1 (Fig. 3B). A more limited number of engrafted donor cells was found in the conditions using direct myoblast bolus injection with VEGF/IGF-1 (Fig. 3D). No transplanted cells were noted in the other

experimental treatments (alginate scaffold VEGF/IGF-1) and control conditions, as expected (Fig. 3A, C). Quantification revealed a 25.5-fold increase in GFP fibers/mm² with scaffolds delivering cells and VEGF/IGF as compared to the bolus transplantation condition (Fig. 3E). Direct epi-fluorescence imaging for GFP on muscle sections harvested at 6 weeks demonstrated a significant population of host muscle continued to be populated by donor cells when transplanted on scaffolds releasing VEGF/IGF-1 (Fig. 3G, J). Conversely, the direct bolus injection of myoblast cells and growth factors led to significantly less engraftment of transplanted cells at this time (Fig. 3H, K), consistent with previous reports using this technique to transplant culture-expanded myoblasts [11,12].

Muscle size and muscle regeneration

Strikingly, a significantly larger skeletal muscle mass was noted in injured muscles treated with scaffolds delivering both myoblasts and growth factors, when compared with the blank alginate at the same time (Fig. 4A), and histologic metrics confirmed an active regenerative process with this treatment. Quantification of the weight of the muscles confirmed a pronounced change with scaffolds releasing cells and VEGF/IGF-1, or VEGF/IGF-1 alone, with statistically significant increases of 46% and 15% (Fig. 4B) respectively, compared with blank alginate at 3 days. The magnitude of these differences decreased with time. Scaffolds delivering GFs led to significant greater muscle mass than blank scaffold at 2 wks and 6 wks, but the scaffolds delivering cells and factors demonstrated an enhancement of muscle mass over all times examined. Representative cross and longitudinal tissue sections of the tibialis muscle at post-treatment 3 days and 6 weeks (Fig. 5A) highlight the increase in centrally located myonuclei in the injured muscles treated with scaffolds delivering cells and VEGF/IGF-1, as compared to the other conditions. To directly analyze muscle regeneration, the numbers of post-mitotic centrally located nuclei per length of myofiber in the resolving muscle tissue were quantified as an index of newly regenerated myofibers (Fig. 5B). At early times post-injury (3 days) the tibialis muscle fibers in the injury group treated with VEGF and IGF-1 without cells showed an approximately 50% increase in centrally located nuclei, as compared with the blank and bolus factors conditions. Alginate delivery of cells with the two factors led to a 78% and a 45% increase in centrally located nuclei per 100 μ m fiber length, as compared with the blank alginate or with scaffold delivering GFs, respectively (Fig. 5B). Significant differences between scaffold delivery of cells and factors and the other conditions were also noted at 6 weeks post-injury, although this metric suggests a diminished level of active regeneration in all conditions by this time point (Fig. 5B).

Differences in muscle fiber type composition were observed under different regeneration conditions 3 days following treatment (Fig. 6). In control limbs, type I and II fibers were observed with type II fibers composing a vast majority of the muscle, a composition consistent with healthy tibialis. A transition in fiber type from type II to type IIC, an intermediate fiber type indicative of active regeneration, was observed with scaffold delivery of VEGF/IGF-1. Smaller populations of type IIC fibers were observed with bolus delivery of VEGF/IGF-1, while type IIC fibers were rarely found in control and blank alginate conditions. In all conditions, the presence of type IIC fibers was transient, as only type II fibers were observed by 2 weeks post-treatment (data not shown). Quantification of the fiber populations confirmed that scaffold delivery of VEGF/IGF-1 led to earlier regeneration, as significant increases in the number of type IIC fibers over the controls were observed only in these conditions (Fig. 6B). Significant numbers of Type I fibers were only observed at 3 days in the control (uninjured tissue) and bolus cell/growth factor delivery conditions.

Limb vascularization and perfusion

The impact of the various conditions on angiogenesis and hemodynamic recovery in the injured tissues was next analyzed. Immunohistochemical analysis of tibialis tissue sections revealed qualitative increases in capillary density based on CD-31 endothelial cell marker staining in both conditions utilizing alginate delivery of growth factors, as compared to the other conditions (Fig. 7A). Quantification revealed that at 6 weeks, VEGF/IGF-1 delivery from the scaffolds resulted in an approximately 1.9 fold and 1.5 fold increase in vessel density in the tibialis muscle, as compared to the ischemic hindlimbs treated with the blank alginate and bolus injection (Fig. 7B). Scaffolds delivering both myoblasts and growth factors induced an even greater increase in blood vessel densities, with a 1.4-fold and 1.2-fold increase compared to scaffolds delivering only growth factors at 3 days and 6 weeks post treatment, respectively. Even this condition, however, did not lead to the same density of capillaries at 6 weeks as is found in control limbs. Bolus delivery of cells and VEGF/IGF only had a modest effect, as compared to the control (Fig. 7A). Laser Doppler Perfusion Imaging (LDPI) indicated improved hemodynamic recovery in mice transplanted with scaffolds delivering both cells and growth factors (Fig. 7C), while delivery of growth factors alone from the scaffolds produced a smaller improvement. In particular, quantification of the perfusion (Fig. 7C) revealed, after the initial, dramatic reduction in blood flow immediately after surgery, a slow spontaneous increase in reperfusion in mice treated with blank alginate and bolus delivery of cells and VEGF/IGF. In contrast, dual VEGF/IGF delivery from the alginate led to a greater increase in tissue perfusion over time, with a final recovery to 75% of the perfusion of the normal limbs at 6 weeks. Mice treated with scaffolds delivering both myoblasts and VEGF/IGF-1 showed a marked increase in blood flow starting from the first week after the injury, reaching 99% of normal perfusion levels at 5 weeks (Fig. 7D).

Contraction force of damaged muscles

To test whether muscle changes induced by scaffold delivery of cells corresponded to increased function, the extent of fibrosis in the muscles and contractile force of the muscles were analyzed. Large quantities of fibrotic tissue resulted, as imaged by Masson's trichrome staining (Fig. 8A), after muscle injury in all conditions. Significant fibrotic tissue remained, even at 6 weeks, in injured muscle tissue treated with either blank alginate or bolus injection of cells and factors, while control uninjured hindlimbs demonstrated little fibrotic tissue. Conversely, limbs treated with scaffolds delivering VEGF/IGF-1 alone and scaffolds delivering both myoblasts and VEGF/IGF-1 exhibited a significant decrease in fibrosis. Quantification of the defect areas (Fig. 8B) showed no statistically significant differences between all treatments at 3 days. However, at 6 weeks after injury, the defects in muscles treated with scaffolds delivering cells and growth factors were significantly smaller than in any other condition. A lesser reduction in defect size was observed in muscles treated with scaffolds delivering VEGF and IGF only, as compared to the blank scaffold treatment. The weight normalized tetanic force generated by the anterior tibialis (Fig. 8C) was measured after maximal tetanic stimulation. At 3 days post-surgery, all experimental conditions resulted in a significant loss of the contractile force, as compared with uninjured control muscle. Muscles treated with blank alginate scaffold demonstrated no recovery over the subsequent 6 weeks. At 2 weeks post-surgery, however, muscles treated with scaffolds delivering VEGF/IGF-1 showed a significant increase in the tetanic force (1.2 fold and 3.2 fold increase in force, respectively, when compared with the control and the blank alginate scaffold). A similar response was observed in animals receiving bolus treatment. A more pronounced effect was measured with scaffolds delivering both cells and VEGF/IGF-1 (2 fold and 1.6 increase in force respectively, when compared with the control and the alginate scaffold delivering VEGF/IGF-1). A decrease toward the control values was observed at the 6 week time-point for all three experimental groups, but animals treated with scaffolds

delivering myoblasts and VEGF/IGF-1 exhibited a greater return of function than the other experimental conditions.

DISCUSSION

Recent studies have shown that significant cell engraftment and effective muscle regeneration results from the transplantation of freshly isolated adult murine satellite cells or entire myofibres, with a 4–40 fold decrease in the regeneration index of tissue culture expanded myoblasts [11–13,26]. The lower regenerative capacity of cultured satellite cell-derived myoblasts has been attributed to a loss of the stem cell subpopulation and/or to a gradual decrease in the cell's proliferative ability during in vitro culture. However, in vitro expansion of human satellite cells will almost certainly be required for significant autologous muscle regeneration in the clinic because of the 4–5 thousand fold greater muscle volume in humans compared to mice, and to the limited number of adult satellite cells obtainable from a biopsy of healthy muscle tissue, as quantified in the Introduction.

The current study demonstrates that myoblasts expanded in vitro can more efficiently drive the muscle reconstitution process when transplanted within an appropriate carrier. The carrier does not serve in this case as a tissue template, but instead mimics certain aspect of the tissue environment immediately surrounding precursor/progenitor cells in injured tissues, migrating into the site of injury and orchestrating the tissue regeneration process.

Localized delivery of VEGF and IGF-1 from alginate scaffolds into the injured muscles was found to enhance muscle regeneration in the severe model of muscle damage used in the present study, as expected from recent findings exploring the combined delivery of VEGF/IGF-1 on the muscle regeneration process in a less severe model of muscle damage [23]. Other studies had also previously demonstrated the utility of a combined delivery of FGF-2 and HGF on muscle regeneration [21], but the VEGF and IGF-1 combination appears to more strongly influence the contractile activity of the skeletal muscle than FGF-2/HGF, and hence its functionality, likely due to the combined effects of VEGF/IGF-1 on muscle regeneration, vascularization and reinnervation [23,30,31].

Transplanting cultured myoblasts with a scaffold that localizes the cells to the injury site long-term, provides adhesive/migration cues, and simultaneously delivering VEGF/IGF-1, dramatically enhanced the participation of these cells in muscle regeneration. Even at early times, a large number of GFP positive cells and GFP-positive muscle fibers were present when the scaffold material was used to deliver the cells and growth factors to the injury site, while very few donor cells engrafted in the bolus delivery treatment. The enhancement in transplanted cell engraftment was accompanied by an increase in the size and weight of regenerating muscles (Fig. 4), as compared with injured muscles treated with either blank alginate scaffolds, or bolus injections of cells/factors, as indicated by increased numbers of centrally located nuclei (Fig. 5). The defect area resolved more rapidly with combined cell and growth factor delivery from scaffolds, as compared with the other treatments that demonstrated larger injured areas characterized by profoundly disorganized and necrotic myofibers (Fig. 7). An increased number of split fibers, and fibers with smaller diameters than those in the non-treated controls further suggest an accelerated regenerative process occurs with scaffold delivery of cells and growth factors, likely due to the contribution of donor cells, via fusion, with existing host myofibers and/or by de novo myogenesis.

During maturation, each myofiber is contacted by a motor neuron which signals the fibers to express a particular type of MHC, leading to fiber type specification [32–34]. In particular, in the tibialis fiber population [33,35] type IIC fibers are classified as undifferentiated and are usually rare in normal adult muscles [33]; but they have been demonstrated to increase

after muscle injury [34]. Without innervation, slow muscles shift toward a faster phenotype while fast muscles shift toward a slower phenotype [36]. Treatment of ischemic muscles with scaffolds releasing both IGF1 and VEGF was found previously to accelerate regeneration of damaged NMJs [23], and this result was likely related to the neuroprotective effects of IGF [31,37–39]. It is not surprising, in light of past work, that the delivery of VEGF and IGF in this study led to a more active regeneration process, as indicated by transient IIC expression in muscle fibers. Significant numbers of type IIC fibers were observed 3 days after injury only in the experimental conditions including VEGF-IGF delivery (Fig. 6A). The rapid appearance and subsequent loss of Type IIC fibers by 2 weeks contrasts with past studies showing a significant increase in type IIC fibers in mouse soleus muscles 2 weeks following cardiotoxin injection [40] and the observation of IIC fibers in regenerating regions of tibialis muscles 30 days after induced injury [37], presumably due to the slower kinetics of regeneration in those studies.

The transplantation of cells from scaffolds delivering growth factors also enhanced angiogenesis and the return to normal tissue perfusion levels, as compared with all the other conditions (Fig. 7). It is perhaps surprising that this condition led to greater effects than the delivery of the factors alone, but may reflect a greater local metabolic need resulting from the enhanced regenerative process driven by the transplanted cells or by endothelial cells possibly present as a low percentage in the transplanted primary cell population.

The dual delivery of cells and growth factors from alginate scaffolds also reduced muscle inflammation and fibrosis (Fig. 8A, B) and most importantly, increased muscle contractile function (Fig. 8C). These results likely relate to the synergic effects played by IGF-1 in modulating inflammation and regeneration processes, and to the pro-angiogenic and neuroprotective effects of VEGF. As reported in this study and elsewhere [23], a significant increase in muscle strength was observed 2 weeks following treatment of injured muscle, followed by a decrease to ~normal value by 6 week post-treatment. This finding suggests a supra-physiologic stimulation of muscle regeneration with this system during the early stages following treatment, followed by a diminishing stimulation that allows the regenerated muscle to remodel to an appropriate level of functionality. The timing of this transition correlates to exhaustion from the scaffold of the growth factors [23], and likely also to a significant decrease in transplanted cell release from the scaffolds. The results of this study demonstrate that the combined delivery of donor cells and growth factors leads to an early and significant increase in the contractile force, as compared to previous work using growth factor delivery alone to promote muscle regeneration [23].

The results of these experiments demonstrate the potential of appropriately designed delivery vehicles to improve the impact of culture-expanded and transplanted cells on the myogenic response in vivo. These effects are likely mediated by the combined action of the ECM components designed into the scaffolds along with the incorporated angiogenic and myogenic growth factors. The heterogeneity of the cell population used in these studies will need to be addressed in future studies, but the approach described here is likely to be broadly useful in the transplantation of many cell types used to promote the regenerative response of multiple tissues.

CONCLUSION

The results of these experiments demonstrate that culture-expanded and transplanted myoblasts can effectively promote skeletal muscle regeneration in vivo if transplanted on an appropriate carrier. Localized delivery of VEGF and IGF-1 from alginate scaffolds into injured muscle enhanced local angiogenesis, altered muscle fiber type, and enhanced muscle regeneration in a severe model of muscle damage. Transplanting cultured myoblasts on

scaffolds that delivered VEGF/IGF-1 dramatically enhanced their direct participation in muscle regeneration, and further enhanced angiogenesis and the return to normal tissue perfusion levels, as compared to growth factor delivery alone. The approach described here is likely to be broadly useful in the transplantation of many cell types used to promote the regenerative response of multiple tissues.

Acknowledgments

We thank the Biological Resources Branch of the National Cancer Institute for providing VEGF for the studies. Supported by National Institutes of Health Grants R01 DE013349 and R43AG029705, the Italian Institute of Technology, the “Fondo degli Investimenti della Ricerca di Base” (Italy), and European Molecular Biology Organization Long-Term Fellowship ALTF 42-2008.

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Figure 1. Schematic representation of the approach

Engineered scaffold containing transplanted cells and growth factors is able to guide tissue regeneration *in situ*.

Figure 2. Experimental design

Wild type C57BL/6J mice were subjected to myotoxin injection, and induction of hindlimb ischemia after 6 days by femoral artery ligation. **(ab)** Tibialis cross-sections, H&E stained before (a) and after (b) muscle injury. **(c)** Primary GFP myoblasts (green), isolated from transgenic Tg(ACTbEGFP)10sb, constitutively expressing GFP in all the cells, were seeded in macroporous RGD-modified alginate scaffold (blue) encapsulating VEGF (red) and IGF-1 (yellow). **(d)** Photograph of the macroporous square-shaped alginate scaffold ($5 \times 5 \times 2$ mm) in a lyophilized form. **(e)** Photograph of scaffold adherent to tibialis muscle at 2 weeks time-point.

Figure 3. Donor myoblast engraftment

Representative images of 3 day post-treatment control limbs (A, C) and injured limbs (B, D) treated with scaffolds (B) or bolus injections (D) delivering cells and VEGF/IGF, showing transplanted myoblast contribution to muscle regeneration. GFP expression (green) by immunofluorescence, in transverse sections of muscle harvested at 3 days after transplantation. (E) Quantification of GFP fibers at 3 days after transplantations, normalized to the total cross-sectional area of each muscle. Values are mean $\pm$ SEM. Representative images 6 weeks post-treatment of control limbs (F, I) and injured limbs (G, H, J, K) treated with scaffolds (G, J) or bolus injections (H, K) delivering cells and VEGF/IGF, showing direct GFP fluorescence (green) on longitudinal (F, G, H) and transverse (I, J, L) cryosections of muscle harvested 6 weeks after transplantations. Size bars correspond to 100 μ m. See also Figures S1 and S2.

Figure 4. Muscle weight and size

(A) Photographs of explanted tibialis anterior muscles at 3 days following treatment with blank alginate (left) and scaffold delivering cells and VEGF/IGF-1 (right). Size bar is shown on the photomicrograph. (B) The weight of the uninjured tibialis muscles (Control) at 3 days, 2 weeks and 6 weeks, compared to muscles after myotoxin/ischemia injury and treatment with blank alginate scaffold, scaffold delivering VEGF/IGF-1, scaffold delivering cells and VEGF/IGF-1, and bolus delivery of cells and VEGF/IGF-1 in PBS. Values represent mean $\pm$ SD ($n=6$) in all graphs. At $*p<0.05$ level the means are significantly different compared with the control and the blank alginate.

Figure 5. Analysis of muscle regeneration

(A) Representative photomicrographs of tibialis muscle tissue sections from injured hindlimbs of C57 mice at post-treatment 3 days and 6 weeks, stained with H&E. Cross and longitudinal sections of injured muscles treated with blank alginate scaffold, scaffold delivering VEGF and IGF-1, scaffold delivering cells and VEGF/IGF-1, and bolus delivery of cells and VEGF/IGF-1 in PBS. (B) The number of centrally located nuclei in muscle fibers at 3 days and 6 weeks post-treatment. Values represent mean $\pm$ SD ($n=6$) in all graphs. ANOVA indicates statistical significance of differences between the different conditions (* $p<0.05$).

Figure 6. Fiber type composition analysis

(A) Fluorescent microscopy images of tibialis muscle fibers at 3 days after treatment with type I fibers (green), type II fibers (red) and type IIC fibers (yellow, colocalization of red and green). Images were taken at 10X magnification. (B) Quantification of type I and type IIC fibers at 3 days after treatment, normalized to the total cross-sectional area of each muscle. Values are mean $\pm$ SD($n=6$).

Figure 7. Quantification of blood vessel densities and hindlimb perfusion

(A) Photomicrographs of tibialis muscles immunostained for the endothelial marker CD-31 at postoperative 6 wks and treatment with blank alginate, scaffold delivering VEGF/IGF-1, scaffold delivering cells and VEGF/IGF-1, bolus delivery of cells and VEGF/IGF-1 in PBS, and control (non-operated) limbs. Size bars correspond to 50 μ m. (B) Quantification of blood vessel densities in tibialis muscles at 3 days and 6 weeks after myotoxin/ischemia injury and treatment with blank alginate, scaffold delivering VEGF and IGF-1, scaffold delivering cells and VEGF/IGF-1, bolus delivery of cells and VEGF/IGF-1 in PBS and, control (non-operated) limb. Values are mean $\pm$ SD ($n=6$). * $p < 0.05$ vs. blank alginate and bolus. (C) Representative color-coded laser Doppler perfusion images (LDPI) at various time points (after surgery, at 3 days, 2, 4 and 6 weeks post-operation) of mice for all conditions. (D) Quantification of LDPI for C57 mice hindlimbs treated with (black square) blank alginate, (gray circle) scaffold delivering VEGF and IGF-1, (gray triangle) scaffold delivering cells and VEGF/IGF-1 and, (black diamond) bolus delivery of cells and VEGF/IGF-1 in PBS. * $p < 0.05$ compared to the blank alginate and bolus; mean values are presented with SD ($n=6$).

Figure 8. Interstitial fibrotic collagen deposition and functional recovery of skeletal muscles

(A) Representative photomicrographs of tissue sections from tibialis muscles stained to highlight the deposition of interstitial fibrotic collagen. Sections were stained with Masson's trichrome, and conditions include tibialis muscles from uninjured hindlimbs (control) and hindlimbs of mice at postoperative 3 days, 2 weeks and 6 weeks treated with blank alginate, scaffold delivering VEGF/IGF-1, scaffold delivering cells and VEGF/IGF-1 and, bolus delivery of cells and VEGF/IGF-1 in PBS. Images are representative of 5 independent experiments. (B) Quantitative analysis of the remaining defect area 3 days and 6 weeks after injury (* $p < 0.05$, as compared with all other conditions; ** $p < 0.05$ compared with blank scaffolds). Values represent mean and SD ($n = 5$). (C) Tetanic force generation of the anterior tibialis muscles of mice was measured at 3 days, 2 and 6 weeks after treatment. Tetanic force was normalized to each muscle's weight to obtain weight-corrected specific force. Stimulation was evoked via parallel wire electrodes with 2.0 ms pulse width and 1 sec train duration, and the maximal stimulation was measured at 15V-300Hz. Mean values are presented with SD ($n=6$); * $p < 0.05$ compared to the other conditions.