Biomaterials 32 (2011) 8905–8914

Contents lists available at SciVerse ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

The role of multifunctional delivery scaffold in the ability of cultured myoblasts to promote muscle regeneration

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ARTICLE INFO

Article history:
Received 28 July 2011
Accepted 8 August 2011
Available online 10 September 2011

Keywords:
Hydrogel
Alginate
Drug delivery
Cell activation
Muscle

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 revascularizaton 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.

1. 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 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 mm^3 [14] while the human TA is 130,000 mm^3 [15]. The regenerative index for subpopulations of non-cultured murine skeletal muscle precursor cells has been reported to lie between 2000 and 4000 (number of donor-engrafted murine muscle fibers generated per 10^5 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 5000 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

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doi:10.1016/j.biomaterials.2011.08.019

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 G_4RGDSP 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.

2. Materials and methods

2.1. 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. [39]. Both alginate polymers were diluted to 1%w/v in double-distilled H_2O, and 1% of the sugar residues in the polymer chains were oxidized with sodium periodate (Aldrich, St Louis, MO, USA), as previously described [39]. 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 G_4RGDSP (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 $CaSO_4$/mL distilled H_2O) at a ratio of 25:1 (40 μl of $CaSO_4$ per 1 mL of 2%w/v alginate solution) using a 1-mL syringe. Alginates were first mixed with recombinant human $VEGF_{165}$ 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 μg/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 × 5 mm were cut using a punch. To produce macroporous 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 μl (200,000 cells/gel) of a cell suspension (4×10^6 cells/ml) was gently poured onto the modified, open-pore polymer scaffolds. The gel were incubated for about 20 min before adding 500 μl of complete culture medium, then maintained at 4 °C prior to animal implantation for ~20 min prior to implantation.

2.2. Myoblast purification, characterization and cultures

Primary myoblasts were derived from 4 to 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 [40]. 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% Pecoll 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 [40]. 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.

2.3. Animals and tissue injury

All animal work was performed in compliance with NIH and institutional guidelines. GFP transgenic mice (C57BL/6-Tg(ACTbEGFP)1Osb) were used as a cell

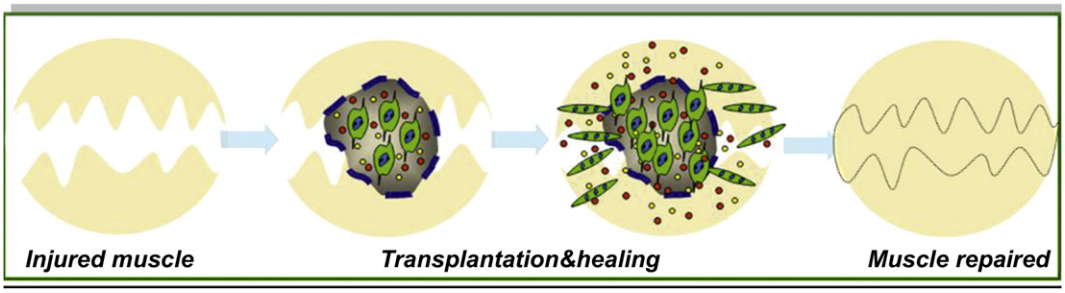

Fig. 1. Schematic representation of the approach. Engineered scaffold containing transplanted cells and growth factors is able to guide tissue regeneration *in situ*.