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Rapid release of growth factors regenerates force output in volumetric muscle loss injuries

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ABSTRACT

A significant challenge in the design and development of biomaterial scaffolds is to incorporate mechanical and biochemical cues to direct organized tissue growth. In this study, we investigated the effect of hepatocyte growth factor (HGF) loaded, crosslinked fibrin (EDCn-HGF) microthread scaffolds on skeletal muscle regeneration in a mouse model of volumetric muscle loss (VML). The rapid, sustained release of HGF significantly enhanced the force production of muscle tissue 60 days after injury, recovering more than 200% of the force output relative to measurements recorded immediately after injury. HGF delivery increased the number of differentiating myoblasts 14 days after injury, and supported an enhanced angiogenic response. The architectural morphology of microthread scaffolds supported the ingrowth of nascent myofibers into the wound site, in contrast to fibrin gel implants which did not support functional regeneration. Together, these data suggest that EDCn-HGF microthreads recapitulate several of the regenerative cues lost in VML injuries, promote remodeling of functional muscle tissue, and enhance the functional regeneration of skeletal muscle. Further, by strategically incorporating specific biochemical factors and precisely tuning the structural and mechanical properties of fibrin microthreads, we have developed a powerful platform technology that may enhance regeneration in other axially aligned tissues.

1. Introduction

Volumetric muscle loss (VML) typically results from traumatic incidents such as those presented from combat missions, where soft tissue extremity injuries are present in 54% of cases, with 53% of these injuries involving soft tissue damage [1,2]. These injuries lead to a devastating loss of musculoskeletal function because of the complete removal of large amounts of tissue, including its native basement membrane [3]. While skeletal muscle has innate repair mechanisms that are largely directed by the native architecture of the basement membrane, it is unable to compensate for large-scale injuries due to the destruction of this regenerative template and growth factor reservoir. The current standard of care for a large-scale skeletal muscle injury is an autologous tissue transfer from an uninjured site [4]. This challenging surgical procedure yields limited restoration of muscle function and can result in complications such as donor site morbidity, infection, and graft failure secondary to tissue necrosis [4,5]. While muscle flaps may be a suitable treatment, as many as 10% of muscle flap surgeries develop complete graft failure, demonstrating the need for an alternative treatment [5,6]. As such, there is a significant need to develop an off-the-shelf, biomimetic scaffold that directs functional skeletal muscle tissue regeneration within large defect sites.

Skeletal muscle regeneration is mediated by local progenitor cells known as satellite cells (SCs), which reside between the sarcolemma and basal lamina of muscle fibers [7,8]. In small muscle wounds such as those from exercise, the basal lamina rapidly releases hepatocyte growth factor (HGF) to stimulate the activation and recruitment of SCs to the wound site [9–12]. Importantly, SC reentry into the cell cycle has been reported to be mediated solely by HGF [13,14]. This immediate, rapid release of HGF occurs in the first 48–72 h of injury, and is important to gather enough progenitor cells to repair large defects [12,15]. However, prolonged HGF signaling will inhibit skeletal muscle regeneration, demonstrating that careful attention must be paid to the time scale that

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HGF is released into a VML defect [12,16].

Acellular scaffolds engineered to direct the regeneration of VML injuries can be tuned via multiple design criteria to facilitate the recruitment of SCs to the injury site and create organized nascent myofibers. A simple method to increase the number of SCs at the injury site is to design scaffolding materials that enable the control release of inductive growth factors upon implantation. Recent studies focus on the revascularization of the tissue [17], or rely on the growth factors present in decellularized extracellular matrix (ECM) materials to direct regeneration [18,19], but they do not address the role of SCs in directing skeletal muscle regeneration. HGF has been used in several studies to enhance the survival of implanted myoblasts, resulting in improved engraftment [20,21]. A separate study incorporated HGF into gelatin scaffolds, and while there was an increase in regenerating myofibers, no functional analyses were performed [22]. While some of these studies report improvements in mechanical function [23], many studies using acellular ECM materials report that long term remodeling, between 3 and 6 months, is necessary for ECM scaffold-mediated functional recovery [24,25]. These data suggest that the addition of an exogenous factor capable of stimulating rapid SC recruitment may facilitate functional recovery at earlier time points, as this would recapitulate regenerative cues missing in VML injuries and expedite the remodeling of the defect site.

An additional limitation of previous acellular strategies is that regenerating myofibers are often not parallel to the native myofibers, limiting the functional efficiency of the newly formed tissue [26–28]. This disorganized regeneration may be a result of the randomly aligned protein networks present in both decellularized ECM and the polymerization process involved in the formation of alginate or gelatin scaffolds. To guide the organized creation of myofibers within an injury site, we developed biomimetic biopolymer microthreads by extruding polymerized solutions of fibrinogen and thrombin into microthread patterns. These aligned fibrin microthread scaffolds have an architectural morphology that is similar to native skeletal muscle tissue and they direct cell alignment along the longitudinal axis of the microthread structure [29]. Results of initial implantation studies investigating the use of fibrin microthreads in a mouse model of VML showed that fibrin microthreads stimulate skeletal muscle regeneration, but noted that a decreased rate of degradation of the microthreads might enhance the microthread-mediated regenerative response [30]. To increase the persistence of microthreads *in vivo*, we developed a crosslinking strategy using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) that attenuates the *in vitro* degradation of fibrin microthreads (EDCn microthreads) without adversely affecting cell attachment, proliferation, or viability, suggesting that these crosslinked materials might be ideal for skeletal muscle regeneration [31].

This study investigated the ability of HGF loaded carbodiimide crosslinked microthreads to enhance regeneration of functional tissue in a mouse VML model. HGF was adsorbed onto EDCn fibrin microthreads (EDCn-HGF) to mimic its *in vivo* release kinetics after injury. We hypothesize that EDCn-HGF microthreads will increase the functional recovery of VML injuries in the *tibialis anterior* (TA) muscle by increasing the number of SCs directed to the injury site. We also examined the impact of HGF delivery on blood vessel formation to determine the ability of these novel scaffolds to promote vascularization of the injury site. The ultimate goal of this modular approach to tissue engineering is to incorporate therapeutic target molecules into morphologically relevant scaffold materials to recapitulate the temporal sequence of regenerative cues essential to enhancing muscle regeneration rather than volume loss or scar formation.

2. Materials and methods

2.1. Microthread preparation

2.1.1. Microthread extrusion

Fibrin microthreads were co-extruded from solutions of fibrinogen and thrombin using extrusion techniques described previously [29,31]. Briefly, fibrinogen from bovine plasma (Sigma, St. Louis, MO; F8630) was dissolved in HEPES (N-[2-Hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]) buffered saline (HBS, 20 mM HEPES, 0.9% NaCl; pH 7.4) at 70 mg/mL and stored at −20 °C until use. Thrombin from bovine plasma (Sigma; T4648) was dissolved in HBS at 40 U/mL and stored at −20 °C until use.

To fabricate microthreads, fibrinogen and thrombin solutions were thawed and warmed to room temperature, and thrombin was mixed with a 40 mM $CaCl_2$ (Sigma) solution to form a working solution of 6 U/mL. Equal volumes of fibrinogen and thrombin/$CaCl_2$ solutions were loaded into 1 mL syringes which were inserted into a blending applicator tip (Micromedics Inc., St. Paul, MN; SA-3670). The solutions were combined in the blending applicator and extruded through polyethylene tubing (BD, Sparks, MD) with an inner diameter of 0.86 mm into a bath of 10 mM HEPES (pH 7.4) in a Teflon coated pan at a rate of 0.225 mL/min using a dual syringe pump. After 10 min, 25.4 cm amorphous fibrin microthreads were removed from the buffer solution and stretched to form three-19-cm microthreads and air dried under the tension of their own weight. Dry threads were placed in aluminum foil and stored in a desiccator until use.

2.1.2. Fibrin microthread crosslinking

Fibrin microthreads were crosslinked using techniques described previously [31]. Briefly, to create microthreads crosslinked in a neutral pH buffer (EDCn), microthreads were hydrated in monosodium phosphate buffer (100 mM NaH_2PO_4, Sigma, pH 7.4) for 30 min and then crosslinked monosodium phosphate or neutral buffer containing 16 mM N-hydroxysuccinimide (NHS, Sigma) and 28 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, Sigma) for 2 h at room temperature. After crosslinking, the buffered EDC/NHS solution was aspirated and the microthreads were rinsed three times in a de-ionized (DI) water bath, air dried, sterilized with ethylene oxide, and stored in a desiccator until use.

2.1.3. Adsorption of HGF to microthreads

Three uncrosslinked (UNX) or EDCn microthreads were attached onto polydimethylsiloxane (PDMS, inner diameter 0.75 in., Dow Corning, Midland, MI) rings, sterilized with 70% ethanol for 90 min, rinsed with DI water three times, and air dried in a laminar flow hood overnight. Sterile microthread-PDMS constructs were hydrated in Dulbecco's phosphate buffered saline (DPBS) and the PDMS surfaces were blocked with 0.25% bovine serum albumin (BSA, Sigma) for 1 h. These solutions were aspirated and replaced with 1 mL of 40 ng/mL or 100 ng/mL of HGF (Peprotech, Rockyhill, NJ) in DPBS and incubated at room temperature for 2 h. The microthreads were rinsed three times in DPBS, twice in L-15 (Mediatech, Inc., Manassus, VA) and immediately used for implantations.

2.2. Cell culture and release of active HGF from microthreads

Immortalized mouse myoblasts (C2C12, ATCC, Manassas, VA) were cultured in a 1:1 (*v/v*) ratio of high glucose Dulbecco's modified Eagle Medium (DMEM, Gibco BRL, Gaithersburg, MD) and Ham's F12 (Gibco) supplemented with 4 mM L-glutamine and 10% fetal bovine serum (FBS, HyClone, Logan, UT). Cells were incubated at 37 °C with 5% CO_2 and maintained at a density below 70%