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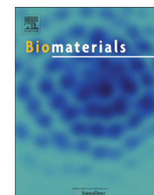

Laminin-111 enriched fibrin hydrogels for skeletal muscle regeneration

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Laminin (LM)-111 supplementation has improved muscle regeneration in several models of disease and injury. This study investigated a novel hydrogel composed of fibrinogen and LM-111. Increasing LM-111 concentration (50–450 μg/mL) in fibrin hydrogels resulted in highly fibrous scaffolds with progressively thinner interlaced fibers. Rheological testing showed that all hydrogels had viscoelastic behavior and the Young's modulus ranged from 2-6KPa. C_2C_{12} myobalsts showed a significant increase in VEGF production and decrease in IL-6 production on LM-111 enriched fibrin hydrogels as compared to pure fibrin hydrogels on day 4. Western blotting results showed a significant increase in MyoD and desmin protein quantity but a significant decrease in myogenin protein quantity in myoblasts cultured on the LM-111 (450 μg/mL) enriched fibrin hydrogel. Combined application of electromechanical stimulation significantly enhanced the production of VEGF and IGF-1 from myoblast seeded fibrin-LM-111 hydrogels. Taken together, these observations offer an important first step toward optimizing a tissue engineered constructs for skeletal muscle regeneration.

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1. Introduction

Skeletal muscle has a remarkable regenerative capacity, primarily due to the reserve pool of muscle resident satellite cells. Satellite cells are the physiologically quiescent muscle stem cells that reside beneath the basal lamina and adjacent to the sarcolemma [1]. The basal lamina is primarily composed of laminin (LM) and collagen type IV. Satellite cells and myoblasts interact with LM through the α7β1 integrin. After damage or mechanical strain, quiescent satellite cells ($Pax7^+$) become activated. Activation of satellite cells leads to self-renewal as well as production of proliferating myoblasts that express MyoD [2]. Differentiation and fusion of myoblasts into myotubes leads to increased expression of myogenin, myosin heavy chain and structural proteins such as α-actinin. The newly formed myotubes fuse together or with existing myofibers to allow for muscle repair. These endogenous mechanisms drive mammalian skeletal muscle repair of moderate injuries (e.g., eccentric, laceration, strain or toxin). However, they are inept in *de novo* regeneration of muscle fiber lost to penetrating trauma [3–11]. Previous studies have shown that increasing angiogenesis or mesenchymal stem cell quantity does not result in effective muscle regeneration following injury [10,12]. Therefore, improving satellite cell function is essential for successful muscle regeneration.

To improve regeneration and function after traumatic skeletal muscle injuries, we propose a focus on LM, a heterotrimeric structural protein in the basal lamina of skeletal muscle fibers that provides an important scaffold for tissue development, maintenance and function. Satellite cells and myoblasts interact with LM through the α7β1 integrin and this interaction is important for the proliferation, adhesion, migration, and differentiation of satellite cells [13–16]. The isoform LM-111 (α1, β1, γ1) is one of the first proteins expressed during embryogenesis and is associated with a variety of biological activities, including stem cell migration, nerve growth, angiogenesis, matrix remodeling and basement membrane assembly [18]. LM-111 supplementation has demonstrated remarkable regenerative capacity in several models of disease [19–21] and injury [22], primarily by influencing satellite cell activity. In mice with congenital muscular dystrophy due to loss of LM α2 chains, LM α1 chains can improve the survival and overall health

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[23,24]. Since activated satellite cells can negatively regulate muscle fibroblasts to inhibit fibrosis development [25–28], we propose that creating a LM-111 rich environment at the site of muscle injury will promote satellite cell activity and suppress fibrotic tissue deposition to improve myofiber regeneration.

Fibrin is an extracellular matrix (ECM) protein that plays a crucial role in the coagulation cascade and serves as a scaffold for tissue repair following injury [29]. Fibrin sealant tissue adhesive is an FDA approved material [30] and fibrin based biomaterials have been successfully used for muscle [31–33] and nerve [34–36] tissue engineering applications. Fibrin allows cells to proliferate, infiltrate and remodel the hydrogel by producing their own ECM proteins within 2–4 weeks [37,38]. Fibrin can also bind cell-derived growth factors that participate in myogenesis [39]. Fibrin can also be isolated easily from patient's own blood thereby preventing the risk of disease transmission and undesirable immune reactions [40]. Moreover, fibrin is a more economical choice compared to collagen and its mechanical properties and degradation rates can be tailored by changing the concentration of the fibrinogen solution or the cross-linker [41]. While LM-111 has been incorporated in several 3D scaffolds [42] including collagen gels [43], poly(ethylene glycol) gels [44] and fibrin-hyaluronic acid gels [45], no study has evaluated the dose response of myoblasts to LM-111 concentrations in a three-dimensional environment. In order to mimic the physiological environment of electroactive and mechanoresponsive skeletal muscle cells, we stimulated the myoblast seeded hydrogels to electromechanical signals *in vitro*. The use of electromechanical stimulation was used to resemble the effects of physical movement in cultured cells. Overall, the goal of this study was to compare fibrin and LM-111 enriched fibrin hydrogels to determine their material properties, potential for myogenesis and response to electromechanical stimulation. A thorough analysis of these hydrogels is essential to determine their potential therapeutic efficacy in traumatic muscle injuries. The results of this study will improve our understanding of myoblast behavior and inform the development of engineered muscle tissues.

2. Materials and methods

2.1. Fibrin gel formation and characterization

LM-111 enriched fibrin hydrogels were created in our lab by combining bovine fibrinogen (20 mg/ml), thrombin (20U/ml), calcium chloride (20 mM), protease inhibitor (1: 100, Sigma-Aldrich) and murine LM-111 (Trevigen). The final concentration of fibrinogen in the gels was 16 mg/ml and that of LM-111 was (50–450 μg/mL) in a 48 well plate. The pure fibrin gels will be referred as FBN and gels containing LM-111 (50–450 μg/mL) will be referred as FBN50, FBN100 and FBN450. To observe the fibrous morphology and 3D architecture of the gels, the fibrin-LM-111 gels were freeze dried, coated with an ultrathin gold layer and observed under a scanning electron microscope (SEM). Fully hydrated fibrin-LM-111 gels were weighed and allowed to dehydrate in a fume hood on weighing paper. The dehydrated weights were recorded after 15 and 30 min of drying. The percentage of mass lost due to dehydration was calculated from the initial weight. For rheological testing, the gels were prepared in a 24 well plate. Frequency sweep was conducted on the AR 2000ex rheometer (flat plate geometry, 0° half angle) at 37 °C to obtain storage (G') and loss moduli (G''). The frequency sweep was performed at 0.1% strain from 1 to 100 Hz at a preload of 0.2 N (n = 3–4). The Young's modulus was calculated from the storage modulus using the following equation, where υ is the Poisson's ratio which is ~0.5 for hydrogels [46,47]:

$$E = G'2(1 + \upsilon)$$

2.2. LM-111 release kinetics

The FBN and FBN450 gels were incubated with 150 μL of PBS at 37 °C for 4 days. The PBS solution was collected, stored at −20 °C and replaced every day for 4 days. 30 μL of the PBS solution collected from the hydrogels was combined with 10 μL of 3× laemmli buffer and resolved on 4–20% Tris-glycine gels at 150 V. LM-111 (1 mg/ml) was used as the control. The proteins were transferred onto a nitrocellulose membrane, which was probed using anti-LM-111 γ1 primary antibody (Millipore), followed by an HRP-conjugated secondary.

2.3. Cell culture

C_2C_{12} myoblasts were seeded on the gels at a density of 100,000 cells/well in a 48 well plate for 4 days (n = 4) in DMEM-F12 media containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (P/S). Cell culture supernatants were collected on day 1 and 4. The production of vascular endothelial growth factor (VEGF), interleukin-6 (IL-6), and insulin-like growth factor (IGF)-1 by C_2C_{12} myoblasts was quantified in cell-culture supernatants using ELISA (Peprotech) as per manufacturer's instructions. Cellular attachment and morphology on the gels were analyzed by fixing cell seeded hydrogels in 4% paraformaldehyde and immunostaining with desmin (Abcam) and DAPI. Images were captured at 40× magnification using a Zeiss Axiocam fluorescent microscope. ImageJ was used for quantification of circularity of myoblasts. Protein lysates from myoblasts were collected on days 1 and 4 and were quantified for myogenic markers using western blotting as previously described [6,10]. Briefly, the gels were rinsed twice in PBS and the cellular protein lysates were collected in RIPA buffer with protease inhibitor cocktail (Sigma). The protein concentration was determined with BSA standards as reference. Equal amounts of reduced and denatured protein (30 μg) were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using 4–20% gels (Bio-rad) and transferred onto nitrocellulose membranes. Equal protein loading was verified by Ponceau S staining. The membranes were probed using anti-desmin (Abcam), anti-MyoD (Millipore) and anti-myogenin antibodies (Millipore), anti-SIRT1 (cell signaling), anti-α-actinin (cell signaling), anti-GAPDH (cell signaling) and HRP-conjugated secondary antibodies.

2.4. Electrical and mechanical stimulation

Myoblasts were seeded on FBN and FBN450 gels at a density of 400,000 cells per well in 4 cm^2 PDMS molds (B-bridge international, n = 3) and allowed to attach for 1 h. The cells were cultured in DMEM-F12 media containing 10% FBS, 10% horse-serum and 1% P/S. 10% horse serum was added to the cells to minimize cellular stress during stimulation and maintain viability. The myoblast seeded molds were subjected to no stimulation (unstimulated controls), electrical stimulation (E stim), mechanical stimulation (M Stim) and synergistic electromechanical stimulation (E + M Stim) to determine if the cells seeded on the gels were responsive to external stimuli. The Ion Optix C-Pace EM tissue culture interface apparatus was used for stimulation. Bipolar rectangular pulse sequences (1 V, 2 ms duration) were used for the stimulation at a frequency of 2 Hz, resulting in an electric field intensity of 50 V/m (Fig. 1). The C_2C_{12} myoblast seeded gels were also subjected to physiological mechanical stimulation (5% strain) in conjunction

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