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Development of a biological scaffold engineered using the extracellular matrix secreted by skeletal muscle cells

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ABSTRACT

The performance of implantable biomaterials derived from decellularized tissue, including encouraging results with skeletal muscle, suggests that the extracellular matrix (ECM) derived from native tissue has promising regenerative potential. Yet, the supply of biomaterials derived from donated tissue will always be limited, which is why the *in-vitro* fabrication of ECM biomaterials that mimic the properties of tissue is an attractive alternative. Towards this end, our group has utilized a novel method to collect the ECM that skeletal muscle myoblasts secrete and form it into implantable scaffolds. The cell derived ECM contained several matrix constituents, including collagen and fibronectin that were also identified within skeletal muscle samples. The ECM was organized into a porous network that could be formed with the elongated and aligned architecture observed within muscle samples. The ECM material supported the attachment and *in-vitro* proliferation of cells, suggesting effectiveness for cell transplantation, and was well tolerated by the host when examined *in-vivo*. The results suggest that the ECM collection approach can be used to produce biomaterials with compositions and structures that are similar to muscle samples, and while the physical properties may not yet match muscle values, the *in-vitro* and *in-vivo* results indicate it may be a suitable first generation alternative to tissue derived biomaterials.

1. Introduction

When provided with the appropriate cues muscle has a robust capacity for self repair. Following mild muscle damage (ex. strains, contusions, and lacerations) cells may be injured, but the underlying extracellular matrix (ECM) is largely intact and repair is robust [1,2]. However, when significant muscle volume is lost (trauma or surgical resection) the physical and chemical cues provided by the ECM are absent and the defect is instead replaced with non-contractile scar tissue [3]. The current standard of care to replace severely damaged or missing muscle is the transfer of autologous muscle flaps. Flap transfers have been used to reconstruct human forearm, elbow, and shoulder muscles [4–6]. Yet, the transfer of muscle tissue is an invasive procedure that causes significant donor site morbidity, and is therefore only rarely indicated. As an alternative to muscle flap transfer, scaffolds or similar bulk implants could be utilized to guide skeletal muscle regeneration within a defect site.

With this goal in mind, several past and recent research efforts have focused on the development of skeletal muscle scaffolds fabricated using synthetic polymers [7–9]. Unlike muscle flaps transfers, synthetic scaffolds can be produced in nearly unlimited quantities and eliminate the complications associated with donor site morbidity. However, the use of synthetic scaffolds for the repair of damaged skeletal muscle has not yet shown clinical promise and appears unlikely to replace flap transfers in the clinical setting. A potential roadblock to the use of synthetic scaffolding for skeletal muscle repair is the foreign body reaction that is directed against all synthetic polymers including degradables. In an effort to evade the foreign body response, more recent efforts have shifted away from synthetic implants and towards tissue derived ECM that can be remodeled by the body's own wound healing machinery [10–13].

Towards this end, the development of ECM scaffolds derived from decellularized skeletal muscle (DSM) tissue has been explored. The multi-molecular properties of tissue derived ECM gives these naturally derived biomaterials a level of complexity and remodelability not observed with synthetic materials [14,15]. In fact, significant research effort has been focused on mimicking the

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properties of ECM in synthetics [16–18], suggesting recognition of the important role of ECM molecules during the wound healing process. Additionally, DSM biomaterials can be produced from allogeneic tissue, thereby eliminating concomitant donor site morbidity. Most importantly, the performance of DSM implants for the repair of skeletal muscle defects has shown promise. When used for the repair of hind-limb muscles in a rat model, decellularized muscle scaffolds were capable of restoring contractile force measurements to 85% of pre-injury levels [19]. Similar encouraging regeneration results have been reported by others using muscle derived ECM scaffolds [20,21]. When viewed as a whole, the tissue derived ECM repair results suggest that these materials can provide the vital cues needed to regenerate volumetric muscle defects. While donated human tissues are the current gold standard we suggest that an engineered ECM (eECM) biomaterial that provides the appropriate regenerative cues by mimicking many of the key properties of DSM is an attractive alternative worth investigation.

To create eECM biomaterials we are exploring methods to farm the ECM that is secreted by populations of cells during growth in culture. While methods to collect isolated molecules from cells in culture have existed for some time, for example hybridoma cells are routinely used to produce antibodies [22], the wholesale collection of cell secreted ECM proteins provides a means to create bulk biomaterials. To collect the ECM that cells secrete, our group has developed a solvent degradable sacrificial foam fabricated from medical grade polyurethane [23]. The scaffold's are used in a manner that is analogous to the use of honeycombs to collect the honey secreted by bees. We have demonstrated that when cells are seeded onto the scaffolds and grown in culture they secrete a multitude of ECM molecules that accumulate within the scaffolds open spaces. The innovative step is that the sacrificial scaffold can be thoroughly dissolved using a water miscible solvent, stripping away the synthetic component and leaving behind only the accumulated ECM. The result is a bulk eECM biomaterial comprised of the molecules secreted by living cells. This report describes the application of our ECM collection process to create an eECM scaffold constructed from the molecules secreted by cultured skeletal muscle cells, as well as comparisons of the material to DSM samples.

2. Methods

2.1. Decellularized skeletal muscle preparation

Gastrocnemius muscle (n = 10) was collected from commercially purchased (Harlan, Indianapolis, IN) Sprague Dawley rats (>300 g) that had been previously euthanized as part of an unrelated study. All animal procedures were performed in accordance with protocols approved by the University of Arkansas Institutional Animal Care and Use Committee (IACUC). Harvested muscle was decellularized following a published protocol [24]. Tissue was rinsed for 90 min in Tris–HCl (10 mM, pH 8.0) with 1% EDTA and 10 kU/ml aprotinin at 4 °C with agitation. Samples were then soaked in 1% sodium dodecyl sulfate (SDS) in Tris–HCL buffer for up to 1 week at room temperature using gentle agitation. To remove nuclear remnants, samples were incubated for 24 h in reaction buffer containing 50 U/mL deoxyribonuclease I and 1 U/mL ribonuclease A in phosphate buffer saline (PBS; pH 7.4) at 37 °C with agitation. Samples were rinsed in PBS, (pH 7.4) for 48 h (4 °C) with multiple rinses to remove any residual decellularization solution, frozen (−80 °C), and lyophilized to preserve tissue structure. DSM samples were weighed and yield was calculated relative to undecellularized muscle volume (mg/cm$^{3)}$).

2.2. eECM sample preparation

Open celled polymeric sacrificial foams were fabricated from a medical grade polyurethane elastomer (Tecoflex, Luibrizol, Wickliffe, OH) using a granular sugar templating process (see Supplemental Data). To form the sugar templates, approximately 5 g of granulated culinary table sugar (sucrose) was mixed thoroughly with 100ul of distilled-deionized (DD) water to create a lightly moistened sugar slurry. The sugar slurry was packed into disk shaped molds (30 mm diameter × 3 mm thickness) and incubated at 50 °C for 20 min to remove the residual water and create a hardened sugar template. Polyurethane (PU) pellets were dissolved in dimethyacetamide (DMAC) (10% w/v) overnight at 50 °C and then pipetted into sugar templates. Polymer soaked templates were immersed overnight in a room temperature DD water bath, which both precipitated the PU solution and dissolved the sugar template. The PU foams were rinsed in DD water at room temperature for 48 h to remove any residual sugar and solvent, frozen (−80 °C), and lyophilized for long term storage.

Commercial rat skeletal muscle myoblasts (L6, ATCC, Manassas, VA) were expanded *in-vitro* following supplier guidelines and seeded onto sacrificial foams at a concentration of 4 million cells per cm^3 of foam. Prior to cell seeding, all sacrificial foams were incubated overnight at 4 °C in a fibronectin solution (20ug/ml) to facilitate cellular attachment. Cell seeded sacrificial foams were maintained in growth media consisting of DMEM-F12 supplemented with 10% FBS, 1 mM ascorbic acid, and transforming growth factor beta1 (TGFβ1, PeproTech, Rocky Hill, NJ) at a concentration 0.5 ng/ml to accelerate cellular ECM production. Cell seeded foams were maintained in culture for 4 weeks. Cultivation conditions utilized during this study were established during preliminary pilot testing (see Supplemental Data). Growth media was exchanged every 48 h. At the completion of the culture period, samples were rinsed in DD water and incubated in DMAC for at least 72 h at room temperature to remove the sacrificial PU foam. The solvent was exchanged 4 times during the 72 h incubation period, twice on the first day and then daily thereafter. The remaining cell derived material was collected, rinsed in DD water, decellularized as previously described, and characterized in comparison with DSM samples.

2.3. Imaging

DSM and eECM samples (n = 5/sample group) were fixed overnight in 4% paraformaldehyde, paraffin embedded, and sectioned with a microtome (10 um). Sections were mounted onto slides, stained with hematoxylin and eosin (H&E), and microscopically imaged (100×). Three representative images from each sample were used to measure porosity (% open space), pore size (mm^2), and pore alignment (average oriented angle) using image analysis software (ImageJ) and guided by published techniques [25,26]. For DSM sample image analysis, the direction of muscle contraction (long axis of the gastrocnemius muscle) was selected to correspond to an orientation angle of 0°. Additionally, bulk eECM and DSM samples, as well as cell free sacrificial foams were lyophilized, sputter coated with platinum, and imaged with the aid of a scanning electron microscope.

To visualize accumulated ECM proteins within DSM and eECM samples, mounted sections were immune-reacted for the presence of collagen type I (αrat collagen 1, mouse IgG1, 750:1, Sigma, St. Louis MO) and cellular fibronectin (αrat cellular fibronectin, mouse IgM, 400:1, Sigma, St. Louis MO) followed by incubation with the appropriate fluorescently labeled secondary antibodies (500:1, Invitrogen, Carlsbad, CA). Sections were counterstained with the nuclear staining reagent DAPI, and then microscopically imaged.

2.4. Mechanical testing

DSM and eECM mechanical properties were measured with the aid of a uni-axial tensile tester (UStretch, CellScale, Ontario, Canada) using techniques familiar to our group [23,27,28]. Prior to testing, samples (n = 4/sample group) were imaged and cross-sectional area was calculated using image analysis software (ImageJ, NIH, Bethesda, MD). Hydrated (PBS, pH = 7.4) samples were deformed at a constant strain rate of 1%/s until failure using a 0.5 N load cell while the load and displacement values were recorded. For each sample, engineering stress versus strain curves were generated from load and elongation data. Strain was determined using grip displacement values. From each curve the tangent modulus was calculated from a linear fit to the stress–strain curve.

2.5. Composition

Collagen content for both DSM and eECM samples was estimated from hydroxyproline concentration. Hydroxy-proline concentration was determined from extracted samples (n = 4/group) using a published technique [29]. Briefly, extracted samples were digested in a 6 N HCL solution (4 h at 110 °C) and then neutralized with sodium hydroxide. Digested samples were mixed with a chloramine T solution (1:2) and incubated at room temperature for 20 min. A dimethyl-aminobenzaldehyde assay solution was added (1:2) and the mixture was incubated at 60 °C for 15 min. During this time a red chromophore develops. Chromophore intensity indicates hydroxy-proline concentration. Sample absorbance was read at 550 nm using a microplate reader. DSM and eECM values were compared against a standard curve, and collagen concentrations were calculated.

To broadly characterize the protein fingerprint, a representative eECM sample was analyzed with tandem mass spectroscopy (MS/MS). The sample was washed with 50 mM ammonium bicarbonate, denatured (Protease Max, Promega, Madison, WI) for 30 min at room temperature, trypsin (20 ng/μl) digested overnight at 37 °C, and purified (Ziptip, Milipore, Billerica, MA). The MS/MS analysis was performed under the direction of the University of Utah proteomic core facility using a hybrid mass spectrometer (LTQ-FT, Thermo Scientific, Waltham, MA). Primary peptide molecular mass spectra were acquired by Fourier transform ion cyclotron resonance. The sequencing of individual peptide spectra was performed by collision induced dissociation in the linear ion trap. Sample proteins were identified by comparison of MS/MS measured peptide sequences to a trypsin-cut specific protein database (Mascot ver. 2.2.1, Matrix Science Inc., Boston, MA).