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The promotion of a constructive macrophage phenotype by solubilized extracellular matrix

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ABSTRACT

The regenerative healing response of injured skeletal muscle is dependent upon a heterogeneous population of responding macrophages, which show a phenotypic transition from the pro-inflammatory M1 to the alternatively activated and constructive M2 phenotype. Biologic scaffolds derived from mammalian extracellular matrix (ECM) have been used for the repair and reconstruction of a variety of tissues, including skeletal muscle, and have been associated with an M2 phenotype and a constructive and functional tissue response. The mechanism(s) behind in-vivo macrophage phenotype transition in skeletal muscle and the enhanced M2:M1 ratio associated with ECM bioscaffold use in-vivo are only partially understood. The present study shows that degradation products from ECM bioscaffolds promote alternatively activated and constructive M2 macrophage polarization in-vitro, which in turn facilitates migration and myogenesis of skeletal muscle progenitor cells.

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1. Introduction

Biologic scaffolds composed of mammalian ECM have been associated with both constructive and functional tissue remodeling in both pre-clinical and clinical studies, including the reconstruction of injured skeletal muscle [1–12]. The mechanisms by which these events are mediated are not completely understood but have been shown to be associated with the recruitment of endogenous stem/progenitor cells [13–22], and modulation of the innate immune response; specifically, enhancement of the M2:M1 macrophage phenotypic ratio [11,23–27].

The adult mammalian response to injury is typically characterized by an immediate pro-inflammatory response with gradual resolution of the inflammatory process including the deposition and organization of scar tissue [28,29]. It has been shown that the transition of macrophage phenotype from the pro-inflammatory M1 form to the alternatively activated and constructive M2 form is required for this resolution phase of inflammation [30,31]. Similarly, although adult skeletal muscle tissue retains inherent regenerative potential following minor injury [32,33], the endogenous repair of skeletal muscle is dependent upon a temporal transition of M1 to M2 macrophage phenotype along with an activated skeletal muscle progenitor cell population(s) [34]. Satellite cell derived myoblasts are the primary stem/progenitor cell-type responsible for the skeletal muscle regenerative response to injury; however, perivascular stem cells (PVSC) found adjacent to blood vessels in skeletal muscle tissue can also give rise to myoblasts [20,35,36]. These skeletal muscle progenitor cells become activated when effector molecules secreted by M1 macrophages promote their mitogenesis [37]. A transition in macrophage phenotype is necessary for the subsequent fusion and differentiation of the expanded progenitor cell population to form myoblasts, myotubes, and mature myocytes. Specifically, M2 macrophage-associated effector molecules facilitate these events [34,38], but the in-vivo mechanisms of the M1 to M2 transition are largely unknown.

The present study examined the effect of degradation products from ECM bioscaffolds, in the form of solubilized small intestinal submucosa (SIS), upon cells associated with the skeletal muscle injury microenvironment. SIS-ECM was used in the present

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study because it has been extensively characterized, represents a clinically relevant biologic scaffold material, and the first publication describing the M1/M2 response to biomaterials evaluated the SIS-ECM bioscaffold [39–42].

Specifically, the objectives of the present study were: (1) to determine the direct effect of ECM degradation products upon macrophage phenotype; and (2) to determine the effect of secreted products from ECM-treated macrophages upon skeletal muscle progenitor cells.

2. Materials and methods

2.1. Overview of experimental design

Primary macrophages were derived from bone marrow of C57bl/6 mice and treated with pro-inflammatory cytokines to derive M1 macrophages, immunomodulatory cytokines to derive M2 macrophages, pepsin-mediated degradation products from porcine small intestinal submucosa (SIS)-ECM to derive Mecm macrophages, or pepsin control buffer. Macrophage supernatants from each group were investigated as potential chemoattractants in a modified Boyden Chamber cell migration assay to study the recruitment of perivascular stem cells (PVSC) and C_2C_{12} skeletal muscle myoblasts. The mitogenic effects of macrophage supernatants were evaluated using 5-bromo-2′-deoxyuridine (BrdU) incorporation. The influence of macrophages upon myogenic differentiation was determined by examining desmin expression.

2.2. Preparation of ECM degradation products

Small intestinal submucosa (SIS) was prepared as previously described [43]. Briefly, the stratum compactum, muscularis mucosa, and tunica submucosa were isolated from the adjacent layers of porcine jejunum (Tissue Source, Lafayette, IN). Following peracetic acid, deionized H_2O, and phosphate-buffered saline treatment to decellularize the tissue, SIS sheets were lyophilized and milled to form a particulate powder. The powder was then solubilized with pepsin as previously described for the preparation of an ECM hydrogel to yield a 10 mg/mL solution [44].

2.3. Isolation and culture of murine bone marrow derived macrophages

Adult, female 6–8-week old C57bl/6 mice obtained from Jackson Laboratories (Bar Harbor, ME) were euthanized via cervical dislocation. Using aseptic technique, the skin from the proximal hind limb to the foot was removed, the tarsus and stifle were disarticulated and the tibia isolated. Similarly, the coxafemoral joint was disarticulated for isolation of the femur. Excess tissue was removed using forceps and a scalpel blade. Bones were kept on ice and rinsed in a sterile dish containing macrophage complete medium consisting of DMEM (Gibco, Grand Island, NY), 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA), 10% L929 supernatant, 0.1% beta-mercaptoethanol (Gibco), 100 U/ml penicillin, 100 ug/ml streptomycin, 10 mM non-essential amino acids (Gibco), and 10 mM hepes buffer. In a sterile environment, the ends of each bone were transected and the marrow cavity flushed with complete medium using a 30-gauge needle. Harvested cells were washed and plated at 10^6 cell/ml, and allowed to differentiate into macrophages for 7 days at 37 °C, 5% CO_2 with complete media changes every 48 h.

2.4. Macrophage polarization and ECM treatment

After 7 days, resulting naïve macrophages were treated with basal media consisting of DMEM, 10% FBS, 100 ug/ml streptomycin, 100 U/ml penicillin and one of the following treatments: (1) 20 ng/ml IFNγ and 100 ng/ml LPS to derive M1 macrophages, (2) 20 ng/ml IL-4 to derive M2 macrophages, (3) 200 ug/ml of SIS-ECM degradation products to derive Mecm macrophages, or (4) 200 ug/ml of pepsin as control buffer. After 18 h of incubation at 37 °C, 5% CO_2 cells were washed with sterile PBS and fixed with 2% paraformaldehyde for immunolabeling.

2.5. Immunolabeling of bone marrow derived macrophages

The primary antibodies used for immunofluorescent staining were: (1) monoclonal anti-F4/80 (Abcam, Cambridge, MA) at 1:200 dilution for a pan-macrophage marker, (2) polyclonal anti-iNOS (Abcam, Cambridge, MA) at 1:100 dilution for an M1 marker, and (3) polyclonal anti-Fizz1 (Peprotech, Rocky Hill, NJ) for an M2 marker. Cells were incubated in blocking solution consisting of PBS, 0.1% Triton-X, 0.1% Tween-20, 4% goat serum, and 2% bovine serum albumin to prevent non-specific binding for 1 h at room temperature. Blocking solution was removed and cells were incubated in primary antibodies for 16 h at 4 °C. After washing in PBS, cells were incubated in fluorophore-conjugated secondary antibodies (Alexa Fluor donkey anti-rat 488 or donkey anti-rabbit 488, Invitrogen, Carlsbad, CA) for 1 h at room temperature. After washing again with PBS, nuclei were counterstained with 4′6-diamidino-2-phenylindole (DAPI) prior to imaging. Images of three 20× fields were taken for each well using a live-cell microscope. Light exposure times for ECM-treated macrophages were standardized based upon those set for cytokine-treated macrophages, which served as a control. Images were quantified using a CellProfiler pipeline for positive F4/80, iNOS, and Fizz1 percentages.

2.6. Validation of macrophage phenotype with western blots

Western blots were performed on treated macrophage cell lysates. Cell lysates were boiled at 100 °C for 5 min and electrophoresed on 15% acrylamide gels. Specifically, 100 ug of protein was loaded into each well. Separated proteins were transferred to PVDF membranes using a wet transfer set up. Following transfer, membranes were blocked for 18 h at 4 °C in TBS-T with 3% milk to prevent non-specific antibody binding. Membranes were incubated in the following primary antibodies for 18 h in 3% milk at 4 °C: (1) polyclonal anti-rabbit mannose receptor (Abcam, Cambridge, MA) at 1:714 dilution for an M2 marker or (2) monoclonal anti-mouse B-actin (Santa Cruz, Dallas, TX) at a dilution of 1:1000 as a loading control. Blots were visualized using a LICOR Odyssey fluorescent imaging scanner. Densitometry of protein expression was standardized to the loading control.

2.7. Progenitor cell culture

Perivascular stem cells isolated from human skeletal muscle and murine C_2C_{12} myoblasts were cultured in DMEM containing 20% FBS and 10% FBS, respectively, 100 ug/ml streptomycin and 100 U/ml penicillin. Cells were grown at 37 °C, 5% CO_2 and were assayed for a chemotactic response in a Boyden chamber when they were approximately 80% confluent.

2.8. Preparation of boyden chamber test articles

Macrophages were cultured in cytokine enriched media as described above for 18 h to promote an M1 or M2 phenotype, or were cultured for 18 h in the presence of solubilized ECM. The media was removed and cells were washed with PBS. Media was then replaced with growth-factor-free DMEM and the cells were incubated for 5 h at 37 °C, 5% CO_2. Supernatant was then collected for use as potential chemoattractant test articles in a Boyden chamber assay. Following supernatant harvest, cells were fixed with 2% paraformaldehyde and stained according to the previously described protocol to verify maintenance of phenotype following growth factor withdrawal.

2.9. Chemotaxis assay

The effect of media conditioned by M1, M2, or ECM-treated macrophages (Mecm) upon C_2C_{12} skeletal muscle myoblast and PVSC chemotaxis was examined using a modified Boyden Chamber cell migration assay. C_2C_{12} myoblasts and perivascular stem cells were cultured in starvation media (DMEM, 0.5% FBS, 1% penn/strep) for 18 h prior to use. Cells were then trypsinized, re-suspended in growth factor-free DMEM, and transferred to a 15 ml conical tube for 1 h incubation at 37 °C, 5% CO_2. Polycarbonate chemotaxis membranes with a pore size of 8 μm were coated with 0.05 mg/ml collagen type I. Macrophage conditioned media (M1, M2, Mecm) or positive (DMEM with 20% FBS) or negative (growth factor-free DMEM) controls were added to the lower wells of a Neuro Probe 48-well micro chemotaxis chamber. Collagen-coated membranes were placed over the chemoattractants and 6×10^5 cells were added to each of the upper wells of the chamber. Cells were allowed to migrate across the chamber for 3 h at 37 °C, 5% CO_2. Following the migration period, non-migrating cells were scraped from the upper side of the membrane using a rubber scraper. Migrated cells that attached to the bottom of the membrane were fixed with 95% methanol and stained with DAPI prior to imaging. Membranes were imaged using a Zeiss Axiovert microscope. The number of migrated cells was quantified using a CellProfiler pipeline.

2.10. Mitogenesis assay

C_2C_{12} skeletal muscle myoblasts and PVSCs were seeded in normal growth media at 1×10^3 cells per well in a 96 well plate. Media was switched to starvation media (DMEM, 0.5% FBS, 1% penn/strep) for 18 h. Following the starvation period, cells were treated with one of the macrophage supernatants, or positive (DMEM with 20% FBS) or negative control (growth factor-free DMEM) media. Treatments were supplemented with 10 uM 5-bromo-2′-deoxyuridine (BrdU) for 18 h for PVSCs and 4 h for C_2C_{12} skeletal muscle myoblasts. Cells were fixed with 95% methanol for 10 min and washed with PBS. Cells were then treated with 2 N HCl for 30 min at 37 °C. Following HCl treatment, cells were blocked using the previously described blocking solution for 1 h at room temperature. Following the blocking period, cells were incubated in G3G4 (Anti-BrdU) antibody (Developmental Studies Hybridoma Bank, University of Iowa) at a dilution of 1:1000 for 16 h at 4 °C. After primary antibody incubation, cells were washed 3 times with PBS and incubated in Alexa Fluor donkey anti-mouse 488 secondary at a dilution of 1:300 for 1 h at room temperature before being subjected to DAPI nuclear stain. BrdU incorporation was imaged using a Zeiss Axiovert microscope and quantified using an ImageJ macro.

2.11. Myogenesis assay

High serum media (20% fetal bovine serum) maintains cell proliferation within the cell cycle and inhibits differentiation. Conversely, low serum media (1% fetal

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