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Biophysical cues enhance myogenesis of human adipose derived stem/stromal cells

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ABSTRACT

Adipose-derived stem/stromal cell (ASC)-based tissue engineered muscle grafts could provide an effective alternative therapy to autografts – which are limited by their availability – for the regeneration of damaged muscle. However, the current myogenic potential of ASCs is limited by their low differentiation efficiency into myoblasts. The aim of this study was to enhance the myogenic response of human ASCs to biochemical cues by providing biophysical stimuli (11% cyclic uniaxial strain, 0.5 Hz, 1 h/day) to mimic the cues present in the native muscle microenvironment. ASCs elongated and fused upon induction with myogenic induction medium alone. Yet, their myogenic characteristics were significantly enhanced with the addition of biophysical stimulation; the nuclei per cell increased approximately 4.5-fold by day 21 in dynamic compared to static conditions (23.3 ± 7.3 vs. 5.2 ± 1.6, respectively), they aligned at almost 45° to the direction of strain, and exhibited significantly higher expression of myogenic proteins (desmin, myoD and myosin heavy chain). These results demonstrate that mimicking the biophysical cues inherent to the native muscle microenvironment in monolayer ASC cultures significantly improves their differentiation along the myogenic lineage.

1. Introduction

Skeletal muscle makes up almost half of the human body mass and is frequently subject to injury [1]. Roughly 35–55% of all sport-related activities and approximately 40% of all traumatic injury involve skeletal muscle damage while muscle tears account for more than 1 million visits to clinics each year globally. In total, this accounts for ca. $6 billion annually worldwide [2,3]. Skeletal muscle possesses robust self-regeneration capacity mainly by the activation of resident satellite cells. However, spontaneous regeneration of volumetric muscle loss (VML) is limited. In such cases, the fibrotic scar tissue formation proceeds more rapidly than *de novo* myogenesis [4]. Eventually, this fibrosis results in the failure of regeneration if the initial muscle loss is more than 20% of the tissue volume [5]. Currently, no effective drug therapies are available to induce healing of VML defects and the surgical treatments using autologous skeletal muscle transplantation (i.e., flaps) are limited by the availability of suitable donor tissues [6]. Numerous efforts have been made to overcome this problem, including blocking pathways of scar tissue formation to stimulate the intrinsic regeneration capacity [7,8] as well as cellular transplantation therapies using satellite cells [9], muscle-derived stem cells [10], bone marrow-derived mesenchymal stem cells (MSCs) [11] and adipose-derived stem cells (ASCs) [12]. However, there are drawbacks to each of these approaches such as deleterious side effects, insufficient incorporation, and rapid cellular clearance from the injury site. For example, when directly injected into the rat soleus muscle, ASCs provided better healing at 2 weeks compared to the untreated group, while no difference was observed at 4 weeks mainly due to insufficient retention at the defect site [12]. To this end, developing tissue engineered muscle grafts from autologous cells could provide an alternative effective therapy.

ASCs are obtained from adipose tissue which is an easily accessible, abundant autologous cell source associated with an acceptable level of patient discomfort, and therefore of great interest for tissue engineering and cell therapy approaches [13–15]. ASCs have demonstrated considerable multi-lineage capabilities, having the ability to differentiate along osteogenic [16], chondrogenic [17], vascular [15], neuronal [18] and myogenic [19] phenotypes. However, there remains room for further exploration of ASCs' myogenic differentiation capacity. Comparative studies have shown that ASCs exhibit better myogenic capacity than MSCs as well as a higher proliferation rate and a greater percentage of stem cell marker expression [20]. Additional reports have explored the myogenic use of ASCs using substrates that mimic muscle niche

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stiffness [21] and alignment [22] as well as blocking negative regulators of myogenesis such as myostatin [23]. While these methodologies all increased the efficiency of differentiation along the myogenic lineage, their overall differentiation efficiency remains relatively low (<15%); thus there remains need for alternative strategies.

One approach is to provide biophysical cues that mimic the physiological stresses acting on the cells *in situ*. Cells residing within physiologically active tissues (i.e. cardiac [24], skeletal and smooth muscle [25,26], tendon [27], ligament [28], cartilage [29], and bone [30]) experience unique forces that govern their native behavior. Mimicking these physical forces within the cellular microenvironment, leads to enhanced tissue specific gene and protein expression as well as structural changes in the extracellular matrix and 3D tissues. For example, application of cyclic strain to primary human myoblasts enhanced elasticity, myofiber diameter and area when compared to static culture conditions [25]. Similarly, enhanced metabolic activity of C2C12 mouse myoblasts were observed in response to 10% cyclic stretch [31] and, conversely, cessation of 12% stretch on C2C12 cultures caused myotube atrophy [32]. Thus, we hypothesized that biophysical cues (cyclic uniaxial strain) in conjunction with biochemical stimuli (azacytidine) would induce ASCs to exhibit enhanced myogenic outcomes (alignment, multi-nucleation, and expression of muscle-specific proteins) and significantly improve the efficiency of differentiation.

2. Methods

2.1. Cell isolation and culture

ASCs were isolated from human subcutaneous adipose lipoaspirates as described previously [15,16] at the Pennington Biomedical Research Center under an Institutional Review Board approved protocol with informed patient consent. Briefly, the lipoaspirate was digested for 1 h at 37 °C in media containing 1 mg/mL Collagenase Type I (Worthington Biochemical Corp., Lakewood, NJ), 10 mg/mL bovine serum albumin (Sigma, St. Louis, MO), and 2 mM $CaCl_2$ in PBS. After centrifugation and re-suspension in Stromal Medium composed of DMEM/F12 Hams Medium (Hyclone, Logan UT) supplemented with 10% fetal bovine serum (FBS; Hyclone) and 1% penicillin/streptomycin/amphotericin (Antibiotic/Antimycotic, Fisher Scientific), the stromal vascular pellet was plated. The adherent population (passage 0) was harvested upon confluence and stored in liquid nitrogen prior to shipment to Johns Hopkins University. Incubation was performed in Culture Medium (CM) containing low-glucose DMEM with 10% FBS and 1% P/S.

2.2. Biochemical and biophysical stimulation

Passage 2 ASCs were seeded at 5000 cells/cm^2 in collagen I coated 6-well plates with flexible membrane substrates (UniFlex Culture Plate, Flexcell International Corp, Hillsborough, NC). Biochemical stimulation was done 24 h after seeding via myogenic induction medium (MIM) applied to the *Induced* group for 24 h. The composition of MIM was determined in preliminary experiments using head-to-head comparisons of several published protocols (data not shown), to include 1% FBS, 5% horse serum (HS; Invitrogen), 10 μM 5-Azacytidine (Aza; Sigma) and 1% P/S within low-glucose DMEM to maximize multi-nucleation. After 24 h, the wells were washed with PBS and fed with fresh CM for the rest of the culture. *Uninduced* cells were retained in CM throughout. ASCs in these two groups were either grown in *Static* or *Dynamic* conditions. *Dynamic* cultures were exposed to uniaxial cyclic strain using the Flexcell system (FX-5000™) between days 3–21 of culture for 1 h/day using 11% strain (the maximum limit of the equipment to provide uniaxial strain) at 0.5 Hz, to approximate the physiological conditions present during normal locomotion [33]. *Static* controls were not subjected to strain.

2.3. Cell morphology and immunocytochemistry (ICC)

The morphology of ASCs was assessed using phase contrast microscopy at days 1, 3, 7, 14 and 21. At 7, 14 and 21 days, the samples were fixed with 3.7% paraformaldehyde, rinsed in PBS and permeabilized with 0.2% triton X-100 for 10 min. After washing with PBS, samples were incubated with 10% normal donkey serum (Sigma) for 30 min to prevent non-specific binding. The samples were then either incubated for 1 h with TRITC-conjugated phalloidin (Sigma) for actin filament staining or primary antibodies rabbit anti-desmin (1:50, Santa Cruz Biotechnology, Dallas, TX), mouse anti-myoD (1:125, Sigma) and mouse anti-skeletal myosin (MHC, Sigma, 1:400) diluted in 1% donkey serum at 4 °C overnight. Following this, samples were incubated with fluorochrome-conjugated secondary antibodies (Alexa Fluor®647-conjugated donkey anti-rabbit IgG or Cy™3-conjugated donkey anti-mouse IgG, 1:200) (Jackson Immunoresearch, West Grove, PA) for 1 h at room temperature. Nuclei were labeled using DAPI. All images were taken using a Zeiss Axio Observer inverted fluorescence microscope. Phase contrast images were used to quantify cell alignment ($n = 24$) while fluorescence images were used for the determination of multi-nucleation (phalloidin-TRITC) and extent of myogenic marker expression (immunostaining for desmin, myoD and MHC; $n = 6$) using Image J software (NIH). Quantification of cellular alignment was performed by measuring the angle of the major axes of the cells relative to the direction of strain, by 4 random angle measurements from 6 different images. Multi-nucleation was assessed by counting the number of nuclei within tubes with a continuous cytoskeleton. Staining intensity measurements were performed using 3 representative images from 2 separate culture wells.

2.4. Statistical analysis

All quantitative results were expressed as means ± standard deviation ($n = 6$). Data was analyzed with statistically significant values defined as $p < 0.05$ based on one-way analysis of variance (ANOVA) followed by Tukey's test for determination of the significance of difference between different groups ($p \leqslant 0.05$).

3. Results

3.1. Biochemical stimulation is necessary to induce ASC multi-nucleation

The effect of biochemical and biophysical stimulation on the morphology and multi-nucleation of ASCs was assessed by actin cytoskeleton staining and ICC at specified time points. Actin staining on day 14 revealed increased cell fusion and multi-nucleation in response to the application of myogenic induction medium (MIM) under *Static* conditions, while *Uninduced* controls proliferated randomly (Fig. 1A). The effect of biochemical stimulation was also prevalent under *Dynamic* conditions; *Induced* cells form multinucleated myotubes by day 14, while no significant multi-nucleation was observed for *Uninduced* counterparts (Fig. 1A). Therefore, biochemical stimulation was found to be necessary to induce ASC multi-nucleation both under *Static* and *Dynamic* conditions and further studies to assess the effect of biophysical stimulation were conducted using *Induced* cells.

The number of myotubes and nuclei/myotube throughout the culture was quantified on days 7, 14 and 21 based on the staining images. The total number of myotubes in *Static* culture was 6.0 ± 1.4 on day 21 with 5.2 ± 1.6 nuclei/myotube (Fig. 1B). Both