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Conditioned medium derived from umbilical cord mesenchymal stem cells regenerates atrophied muscles

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ABSTRACT

We investigated the regenerative effects and regulatory mechanisms of human umbilical cord mesenchymal stem cells (UC-MSCs)-derived conditioned medium (CM) in atrophied muscles using an in vivo model. To determine the appropriate harvest point of UC-CM, active factor content was analyzed in the secretome over time. A muscle atrophy model was induced in rats by hindlimb suspension (HS) for 2 weeks. Next, UC-CM was injected directly into the soleus muscle of both hind legs to assess its regenerative efficacy on atrophy-related factors after 1 week of HS. During HS, muscle mass and muscle fiber size were significantly reduced by over 2-fold relative to untreated controls. Lactate accumulation within the muscles was similarly increased. By contrast, all of the above analytical factors were significantly improved in HS-induced rats by UC-CM injection compared with saline injection. Furthermore, the expression levels of desmin and skeletal muscle actin were significantly elevated by UC-CM treatment. Importantly, UC-CM effectively suppressed expression of the atrophy-related ubiquitin E3-ligases, muscle ring finger 1 and muscle atrophy F-box by 2.3- and 2.1-fold, respectively. UC-CM exerted its actions by stimulating the phosphoinositol-3-kinase (PI3K)/Akt signaling cascade. These findings suggest that UC-CM provides an effective stimulus to recover muscle status and function in atrophied muscles.

1. Introduction

Skeletal muscle atrophy is caused by various conditions of muscle disuse (e.g., spaceflight, immobilization, long bed rest, and denervation) and leads to adverse physiological and functional consequences. These physiological changes also bring about functional decline, including muscle weakness and fatigue, as well as a significant reduction in motor activities (Adams et al., 2003). The soleus muscle, an important anti-gravity slow-twitch muscle, is especially likely to be affected by decreased muscle use (Fitts et al., 2000). Because the soleus muscle is critical for maintaining posture and balance, preserving its activity is essential to safeguard against functional depression of the muscle (Eberstein and Eberstein, 1996).

To date, various types of therapies have been employed for curing or preventing muscle atrophy, encompassing treadmill training (Gwag et al., 2009), resistive exercise (Schneider et al., 2003), electrical stimulation, (Guo et al., 2012), and chemical treatment (Chan et al., 2005). However, these regimens are not applicable to certain cases (e.g., patients undergoing prolonged bed rest, wheelchair users, elderly subjects, and other relatively inactive individuals). Recently, mesenchymal stem cells (MSCs)-based therapies have attracted increasing attention as promising treatments for muscle-related diseases (Ichim et al., 2010; Kim et al., 2015; Meregalli et al., 2013). According to many investigators, the regenerative mechanisms of MSCs are mainly due to paracrine factors secreted by the cells at the transplanted site, rather than to the differentiation/cell replacement capacity of the MSCs themselves (Caplan and Dennis, 2006; Di Santo et al., 2009).

MSCs-derived paracrine factors (cytokines, growth factors, extracellular matrix components, exosomes, and anti-oxidant molecules) are found in the conditioned medium (CM)/secretome obtained from cultured cells. These factors trigger intrinsic initiation of tissue repair. In particular, vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), and fibroblast growth factor (FGF)-7 play regulatory roles in the growth and differentiation of muscle cells (Hayashi et al., 2004; Messina et al., 2007). Moreover, macrophage inflammatory protein 2 (MIP-2), monocyte chemotactic protein 1 (MCP-1), and activin A can recover skeletal muscle functions via activation of satellite cells (Fukada et al., 2007; Washington et al., 2011). Although the regenerative potential of MSCs-CM against muscle disease is based on the identified

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functions of individual MSCs-CM-derived factors, relevant studies mainly focus on muscle trauma and muscular dystrophy (Cerletti et al., 2008; Shi et al., 2009). Thus, verification of the therapeutic effects and action mechanisms of MSCs-CM is required for the overall evaluation of the restorative impact of the secretome in atrophied muscles.

We previously assessed the regenerative influence of various MSCs on degenerated muscle using a hindlimb suspension (HS)-induced muscle atrophy model in rats (Kim et al., 2015). Direct injection of MSCs into the muscle atrophy model significantly promoted the recovery of damaged muscles and also increased the expression levels of muscle-related proteins and genes. Among the investigated MSCs, human umbilical cord-derived MSCs (UC-MSCs) showed the greatest efficacy in all analyzed parameters, including muscle regeneration and downregulation of atrophy-related factors. Therefore, we hypothesized that paracrine factors secreted from UC-MSCs might likewise regenerate atrophied muscles and promote the expression of muscle-specific regenerative factors. To evaluate this hypothesis, the current study assessed culture duration-dependent cytokine and growth factor content in the CM to determine the optimal time point for harvesting the medium. The salutatory actions of the UC-MSCs-derived CM (UC-CM) on functional changes in the soleus muscle were then determined in a rat model. Finally, we assessed the ability of UC-CM to stimulate the phosphoinositol-3-kinase (PI3K)/Akt pathway, an important pathway associated with protection against muscle atrophy.

2. Materials and methods

2.1. Preparation of UC-MSCs

This study was approved by the Institutional Review Board of CHA University (Seongnam, Korea; IRB No. 201412-BR-003-02). The procedures for UC-MSCs isolation and culture were described previously (Kim et al., 2013). Briefly, to isolate UC-MSCs, Wharton's jelly was sliced into 5-mm explants after removing the umbilical vessels, and the slices were subsequently attached and cultured in T-175 culture flasks (SPL Life Sciences Co., Pocheon, Korea) in Minimum Essential Medium Eagle Alpha Modification (α-MEM; Hyclone Laboratories, Inc., Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone) and 100 mg/mL streptomycin plus 100 IU/mL penicillin (Hyclone). UC-MSCs were used at passage 4–5 for all experiments. Cells were maintained at 37°C in a humidified incubator with an atmosphere of 5% CO_2 (v/v), and the medium was replaced every 3 days.

2.2. Cell proliferation analysis

UC-MSC proliferation was evaluated under serum-free conditions using the WST-1-based, colorimetric Cyto X™ Cell Viability Assay Kit (LPS solution, Daejeon, Korea). UC-MSCs were seeded into 96-well culture plates (SPL; 1×10^3 cells/well). After 24 h, non-adherent cells were washed away with Dulbecco's phosphate-buffered saline (DPBS; Hyclone), and the medium was replaced with fresh serum-free α-MEM. At the indicated time points (6, 12, 24, 48, and 72 h), WST-1 solution (20 μL) was added into each well (final dilution = 1:10), and the reaction mixture was incubated at 37°C for 2 h. Sample absorbance was measured at 450 nm using an Epoch Spectrometer/microplate reader (BioTek Inc., Winooski, VT, USA). The mean and standard deviation (SD) values were obtained from three independent experiments.

2.3. UC-CM collection

UC-MSCs were seeded at a density of 2×10^6 cells in T-175 culture flasks. When the cells reached sub-confluence (80–90% confluency), they were gently washed twice with DPBS, and the medium was replaced with fresh serum-free α-MEM. Culture medium was harvested from the cells 24 and 48 h later, and the collected medium was centrifuged at 1500 rpm for 5 min. The supernatants were used as CM samples. The CM was lyophilized using a freeze dryer (FDU-8612; Operon Co., Gimpo, Korea), and the desiccated matter was placed into a 5 mL sterile glass vial and stored at 4°C until use. The CM was resuspended in normal saline (0.9% w/v) for use in in vivo experiments.

2.4. Cytokine assay

Protein profiles in the UC-CM were analyzed for 507 growth factors, cytokines, and other active factors using the RayBio® Biotin Label-based Human Antibody Array I Kit (Ray Biotech, Inc., Norcross, GA, USA). Briefly, the prepared samples (UC-CM) were mixed with the provided biotin-labeling reagent and incubated with gently shaking for 30 min at room temperature. Then, the provided stop solution was added into reaction solution and the samples were centrifuged at 1500 rpm for 3 min to remove any unbound biotin using spin column. The column were washed with 0.1 M PBS (Biosesang Inc., Seongnam, Korea), the biotin-labeled samples were collected and were stored at −80°C until use. The array membranes were blocked by incubation with blocking buffer for 1 h at room temperature and then incubated with biotin-labeled UC-CM (1 mL) for 2 h at room temperature. Samples were washed twice with the provided wash buffer, and the membranes were incubated with horseradish peroxidase-conjugated streptavidin for 2 h at room temperature (Kim et al., 2006). Chemiluminescence signals on the array membranes were detected using a LAS-3000 Imaging System (Fujifilm Inc., Tokyo, Japan), and the signal intensity of each spot was quantified using Multi Gauge V3.0 Software (Fujifilm).

2.5. In vitro cell migration assay

To investigate the pro-migratory capacity of UC-CM toward muscle cells, isolated L6 rat skeletal muscle cells were seeded at a density of 5×10^5 cells/well in a six-well plate, allowed to adhere, and grown to 80–90% confluency in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS. Subsequently, vertical wounds were created using a sterile pipette tip, and cellular debris was removed by washing with PBS (Biosesang Inc.). The cells were incubated with DMEM plus 10% FBS (positive control), serum-free DMEM, or serum-free DMEM supplemented with UC-CM (1 mg/mL) obtained at 24 and 48 h of culture, respectively, at 37°C in an atmosphere of 5% CO_2. Migrating cells were observed via light time-lapse microscopy with an ECLIPSE E600 microscope (Nikon, Fukuoka, Japan) at a magnification of 200×.

2.6. Experimental animals and HS-induced muscle atrophy model

All procedures for animal care and treatment were performed in accordance with the Care and Use of Laboratory Animals Guidelines of CHA University, and all protocols were approved by the CHA University Committee of Animal Experiments (Approval No. 130012). The HS-induced muscle atrophy model was generated as previously described (Kim et al., 2015). Briefly, female Sprague-Dawley rats (200–220 g; 5 weeks of age; n = 10 animals per group) were purchased from Koatech Co. Ltd (Pyeongtaek, Korea). The rats were housed at a controlled temperature of 24°C, a relative humidity of 55%, and a 12 h/12 h light/dark cycle. Animals were randomly divided into the untreated control group or the HS-induced muscle atrophy group. To induce muscle atrophy, a HS apparatus was utilized to lift the back legs off the ground at a 30° angle, and HS conditions were continued for 2 weeks. After 2 weeks of HS, the HS group was divided into the following subgroups: 1) saline injection