Low-Intensity Ultrasound (LIUS) Attenuates Myogenic and Adipogenic Differentiations of Rat Bone Marrow-Derived Mesenchymal Stem Cells (MSCs)

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Abstract: Mesenchymal stem cells (MSCs) are multipotent stem cells that can differentiate into a variety of cell types, including chondrocytes, osteocytes, adipocytes, myocytes, and neurons. However, more efficient methods for specific lineage differentiation from MSCs are still required for their clinical application. In the present work we examined the effects of low intensity ultrasound (LIUS) on myogenic and adipogenic differentiation of MSCs isolated from the bone marrow of rats. We found that the LIUS stimulation has inhibitory effects on both differentiation toward myocyte and adipocyte from rat MSCs (rMSCs). bFGF enhanced the myogenic and adipogenic differentiation when MSCs were cultured in each differentiation medium, inducing the mRNA expression of myogenic and adipogenic markers. The LIUS stimulation showed the decrease in the expression of myogenic markers, desmin and troponin C, in the condition of myogenic differentiation containing bFGF. Similarly, the LIUS stimulation also showed the decrease in the expression of PPARγ and the Oil Red O staining in adipogenic differentiation. These results reveal that mechanical stimulation by LIUS attenuates both myogenic and adipogenic differentiation of rMSCs in vitro.

Key words: low intensity ultrasound, myogenic differentiation, adipogenic differentiation, mesenchymal stem cells, mechanical stimulation

1. Introduction

Recent studies have reported that mesenchymal stem cells (MSCs) are undifferentiated multipotent cells capable of differentiating into several cell types such as osteoblast, chondrocytes, myocytes, adipocyte and even into neural cells, and can be obtained from many accessible sources, such as bone marrow, adipose tissue, placenta and umbilical cord. The specific lineage differentiation of MSCs is mainly influenced by culture condition, notably growth factors and some chemicals, in vitro. Growth factors that have regulatory effects on MSCs include the transforming growth factor beta (TGF-β) family, the insulin-like growth factors (IGF), the epidermal growth factor (EGF), and the vascular endothelial growth factor (VEGF). Chemicals, also play important roles in regulating the MSCs differentiation. For example, treatment of MSCs with dexamethasone, isobutyl methyl xanthine, indomethacin and insulin preferentially promote their adipogenic differentiation by regulating the several adipogenic transcription factors, including CAAT/enhancer binding proteins-α (C/EBPα) and peroxisome proliferator-activated receptor γ (PPARγ). The chemical compound AZA, which causes extensive demethylation of 5-methylcytosine and reduces DNA methyltransferase activity in the cell, has been used in several studies to induce the differentiation of MSC into muscle cells such as cardiomyocytes and urethral sphincter. Although a large number of studies have assessed the protocols to induce MSCs into the desired specific fate, the molecular mechanisms underlying growth factor and chemical-induced regulatory signals are not well understood.

In addition to growth factors and chemicals, mechanical stimulation also plays an important role in the proliferation and differentiation of a variety of cell types by regulating mechanical signals. Previous studies have reported that various mechanical stimuli such as stretch, shear stress, hydrostatic...
pressure, tension and ultrasound (US) can induce metabolic activity of cells, and particularly the expression of extracellular matrix (ECM) genes.\textsuperscript{14,15} Besides, the effects of mechanical stimulation on the differentiation of embryonic and adult stem cells has been reported.\textsuperscript{16,17} Therefore, it seems that the mechanical stimuli can regulate activity of stem cells in many ways, but the exact mechanism of their signaling pathways remains to be determined. Ultrasound is a special type of sonic waves with high frequency above the limit of human audibility of 2-20 kHz. The frequency of ultrasound, around 3-10 MHz, is used in general in clinical purposes, such as diagnostic and therapeutic devices. Also, LIUS is an acoustic energy with non-thermal effects, and has a low intensity (less than 1 W/cm\textsuperscript{2}) ultrasound. It was known to improve fracture healing and increase the mechanical strength of fracture callus in clinical studies and animal models,\textsuperscript{18} and to enhance the repair of articular cartilage in cartilage defect models.\textsuperscript{19-21} LIUS stimulation also enhanced the synthesis of ECM proteins, such as collagen type II and proteoglycans according to the culture condition and the ultrasound intensity in cultured chondrocyte.\textsuperscript{22-25} In addition, it has shown to induce the changes in cell membrane permeability by the formation of temporary pores in the cell membrane,\textsuperscript{26} and recovery of various connective tissues such as the bone,\textsuperscript{27} muscle,\textsuperscript{28} and tendon.\textsuperscript{29} Unlike bone and cartilage injuries, there are still no scientific evidence and consensus statement on the therapeutic effects of LIUS for the treatment of muscle injuries. Our recent study revealed that the LIUS stimulation enhances chondrogenic differentiation in alginate culture with rabbit MSCs, showing the expression of chondrogenic markers such as collagen type II, aggrecan, and Sox-9. Furthermore, the effects of LIUS on the expression of chondrogenic markers were observed independently of TGF-\(\beta\)3 treatment, a well-known inducer of chondrogenesis.\textsuperscript{30} Little is known, however, about the effect of LIUS on the differentiation potential of other cell lineages of MSCs in vitro. Thus, we investigated the mechanical effect of LIUS on the myogenic and adipogenic differentiation of bone marrow-derived MSCs by examining the proteins and mRNA expression of genes related to these differentiation processes.

2. Materials and Methods

2.1 Isolation of rMSCs

Femurs and tibias of 4-week-old male Sprague Dawley rats were excised and connective tissue was removed.\textsuperscript{30} Briefly, marrow cells were washed twice to remove the fatty components, and resuspended in Dulbecco's phosphate-buffered saline (DPBS, Gibco). The samples were layered on Ficoll-Paque (Amersham Pharmacia Biotech) and centrifuged 30 min at 2,000 g to concentrate nucleated cells at the interface. This fraction was collected, washed once with \(\alpha\)-MEM media (Sigma), and resuspended in tissue culture medium. The primary cultures of these cells were established at 5\times10\textsuperscript{5} cells/cm\textsuperscript{2}, and the non-adherent cells were removed after 6 days. After the cells had grown to near confluence, they were passaged two to three times through digestion with 0.25\% trypsin and 0.02\% EDTA. The third-passage cells were cultured with \(\alpha\)-MEM supplemented with 100 IU/mL of penicillin G, 100 mg/mL of streptomycin and 10\% FBS (Gibco, Invitrogen).

For differentiation experiments, the cells at passage 4 or 5 were used.

2.2 Myogenic and Adipogenic Differentiation

rMSCs were seeded onto 60-mm culture dishes at 2\times10\textsuperscript{4} cells/dish and allowed to recover for 1 day. To induce myogenic differentiation, these cultures were incubated for 24 h with differentiation medium containing 10 \(\mu\)M 5-azacytidine (Sigma, St-Louis, MO, USA) and 50 \(\mu\)M hydrocortisone (Sigma, St-Louis, MO, USA) as described by Wakitani et al.,\textsuperscript{31} and treated with or without 10 ng/ml bFGF (R&D, USA) for 14 days. To induce adipogenic differentiation, rMSCs were cultured in an adipogenic differentiation medium consisting of the complete medium supplemented with 1 \(\mu\)M dexamethasone, 0.5 \(\mu\)M methyl-isobutylxanthine, insulin (10 \(\mu\)g/mL) and 100 \(\mu\)M indomethacin,\textsuperscript{32} and then treated with or without 10 ng/ml bFGF for 14 days.

2.3 Stimulation with LIUS

To stimulate rMSCs with LIUS, the Noblelife\textsuperscript{\textregistered} apparatus (Duplogen, Suwon, Korea) was used, which has three transducers of 5 cm in diameter and controllers to modulate an output intensity and treatment time. The LIUS stimulation was performed for 10 min every day at a frequency of 1 MHz and an intensity of 100 mW/cm\textsuperscript{2} in a continuous wave fashion. The operation of the transducers was checked before each experiment. The culture dishes were placed on ultrasound transducers using a coupling gel. After the LIUS stimulation, the cells were incubated for 14 days for differentiation study.

2.4 Immunocytochemistry

To characterize the differentiation of rMSCs, we performed immunocytochemistry analyses. After 7 and 14 days of each induction, the cells on the cover glass were fixed with 4\% paraformaldehyde for 5 min at room temperature, and then incubated sequentially with primary and FITC-conjugated secondary antibodies for 1 h each at room temperature. The