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Low-intensity pulsed ultrasound regulates proliferation and differentiation of osteoblasts through osteocytes

Lei Li [a], Zheng Yang [a], Hai Zhang [b], Wenchuan Chen [a], Mengshi Chen [c], Zhimin Zhu [a,*]

[a] State Key Laboratory of Oral Diseases, West China Hospital of Stomatology, Sichuan University, Chengdu, China
[b] Department of Restorative Dentistry, School of Dentistry, University of Washington, Seattle, WA, USA
[c] Department of Biomechanics, Sichuan University, Chengdu, China

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ABSTRACT

Low-intensity pulsed ultrasound (LIPUS) has been used as a safe and effective modality to enhance fracture healing. As the most abundant cells in bone, osteocytes orchestrate biological activities of effector cells via direct cell-to-cell contacts and by soluble factors. In this study, we have used the osteocytic MLO-Y4 cells to study the effects of conditioned medium from LIPUS-stimulated MLO-Y4 cells on proliferation and differentiation of osteoblastic MC3T3-E1 cells. Conditioned media from LIPUS-stimulated MLO-Y4 cells (LIPUS-Osteocyte-CM) were collected and added on MC3T3-E1 cell cultures. MC3T3-E1 cells cultured in LIPUS-Osteocyte-CM demonstrated a significant inhibition of proliferation and an increased alkaline phosphatase activity. The results of PGE_2 and NO assay showed that LIPUS could enhance PGE_2 and NO secretion from MLO-Y4 cells at all time points within 24 h after LIPUS stimulation. We conclude that LIPUS regulates proliferation and differentiation of osteoblasts through osteocytes in vitro. Increased secretion of PGE_2 from osteocytes may play a role in this effect.

1. Introduction

As an established therapy for fracture repair, low-intensity pulsed ultrasound (LIPUS) has demonstrated an ability to accelerate healing of fresh fractures, to minimize delayed healing and to stimulate healing of established nonunions in animal studies and clinical trials. Using rats with artificial femoral fracture, Azuma et al. have shown that LIPUS increases the rate of fracture healing at all stages of the repair process [1]. The clinical application of LIPUS has been evaluated in a number of prospective, randomized, double-blind, placebo-controlled trials on several types of bone fractures, in which LIPUS accelerated recovery of bone strength following fracture [2,3] and showed efficacy in treatment of established nonunions [4,5].

Even though effects of ultrasound are evident, the biophysical mechanisms remain unclear. LIPUS has been shown to upregulate osteocalcin and insulin like growth factor I (IGF-I) expression in ST2 cells of bone marrow origin and stimulate differentiation and mineralization of osteoblasts [6–8]. Exposure of LIPUS leads to an increase of prostaglandin E_2 release and an upregulated expression of PGE synthesis enzyme cyclooxygenase-2 in mouse osteoblasts [9]. Enhanced synthesis of collagen was found in fibroblasts after exposure to ultrasound [10,11]. Zhang et al. in a cell culture study with chondrocytes from chicken embryos observed increased proliferation of undifferentiated chondrocytes after LIPUS treatment [12].

Osteocytes residing in the lacunar–canalicular system are the most abundant common cells in bone. The large population, ideal location and unique morphologies make osteocytes ideal candidates for detection of external stimulations and generation of signals that affect osteoblasts, osteoclasts and their progenitors in the bone marrow, and thus orchestrate local bone formation and resorption [13,14].

Therefore, we hypothesize that ultrasonic stimulation is detected by osteocytes and then translated into certain biochemical signals that in turn regulate the activities of the effector cells including osteoblasts, osteoclasts, MSC and so on. In the current study, we first investigated effects of conditioned medium from osteocytes to proliferation and differentiation of osteoblasts, and then measured PGE_2 and NO secretion from osteocytes stimulated by LIPUS.

2. Methods

2.1. MLO-Y4 and MC3T3-E1 cell cultures

MLO-Y4 osteocytic cell line was a kind gift from Professor Lynda Bonewald (School of Dentistry, University of Missouri, Kansas City, MO). MLO-Y4 cells were cultured in a-modified minimal essential

* Corresponding author. Address: State Key Laboratory of Oral Diseases, No. 14, 3rd Section South Renmin Road, Chengdu, Sichuan 610041, China.
E-mail addresses: geraldleelei@163.com (L. Li), hxzhimin@163.com (Z. Zhu).

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medium (α-MEM, GIBCO) supplemented with 5% fetal bovine serum (FBS, GIBCO), 5% calf serum (CS, HyClone) and antibiotics (penicillin/streptomycin, GIBCO). Osteoblastic MC3T3-E1 cells were maintained in α-MEM supplemented with 10% FBS and antibiotics.

2.2. Conditioned medium from LIPUS-stimulated osteocytes

Ultrasound was generated with the transducer for a SAFHS (Sonic Accelerated Fracture Healing System, NexSound, China) operated at a 1.5-MHz frequency in a pulsed-wave mode. MLO-Y4 cells were seeded in 3.5-cm-diameter dishes at a density of 6×10^5/dish. After one day, 3 ml of fresh medium in each dish was changed 1 h prior to exposure of LIPUS. The dish was placed on an ultrasound absorbing rubber pad with gel applied between them. The sterilized transducer was immersed vertically into the culture and just touched the surface of the medium. The distance between the transducer and the cells was approximately 3 mm. After the whole apparatus was set up in an incubator, the cells were exposed to LIPUS for 20 min. Then the conditioned medium from LIPUS-stimulated MLO-Y4 (LIPUS-Osteocyte-CM) was collected after 1 h. A sham transducer without emission of LIPUS was used for the control conditioned medium (Osteocyte-CM) in the same number of dishes.

2.3. MTT assay

MC3T3-E1 cells were seeded in five 96-well plates at a concentration of 1×10^4/well. After 1 day, the medium of respective wells was changed with 200 μl of LIPUS-Osteocyte-CM, Osteocyte-CM or fresh medium. Then after 0, 1, 2, 3 and 4 days, proliferation of MC3T3-E1 cells in one plate was measured. The assay was an estimate of *succinate dehydrogenase* (SDH) activity through the MTT method. Twenty microliters of MTT solution (5 mg/ml, Sigma) was added to each well. After incubation with the MTT solution for 3.5 h, cells were washed with PBS gently. PBS was removed and 150 μl/well of DMSO solution was added to dissolve formazan. The blue color of the dissolved formazan was read in a plate reader at 570 nm.

2.4. Measurement of alkaline phosphatase (ALP) activity

Ascorbic acid (50 μg/mL) (Sigma) and 10 mM β-glycerophosphate (Sigma) were added to the medium to induce differentiation of MC3T3-E1 cells. MC3T3-E1 cells were seeded in 12-well plates at a concentration of 1×10^5 /well. The medium of respective wells was changed with LIPUS-Osteocyte-CM or Osteocyte-CM after 1 day. The medium was changed every three days. At day 10, cell layers were gently rinsed three times with PBS and lysed with 0.2% TritonX-100 in saline. The lysates were then centrifuged for 5 min at 12,000*g* prior to collection of the supernatant containing the alkaline phosphatase. The activity of ALP in cell lysates was measured using ρ-nitrophenylphosphate (PNPP) as the substrate. Absorbance of the enzyme products at 405 nm was measured at 1 and 4 min after mixture of samples and reagents. Estimation of protein content was carried out using a BCA Protein Assays Kit (Pierce). The activity of ALP was expressed as difference of optical density at two time points normalized by protein concentration.

2.5. PGE_2 and NO assay

MLO-Y4 cells were seeded in 3.5-cm-diameter dishes at a density of 6×10^5/dish. After 1 day, 3.2 ml of fresh medium was changed 1 h prior to LIPUS exposure. The cells were exposed to LIPUS for 20 min. Two hundred microliters of medium was collected from the dish before LIPUS exposure and at 0, 1, 6, 12 or 24 h after LIPUS exposure. A transducer without emission of LIPUS was used for control groups. The transducers were kept in the same distance from the cells as described above.

NO was measured as nitrite accumulated in the medium using Griess reagent. Serial dilutions of $NaNO_2$ in the medium were used to obtain a standard curve for each measurement. The absorbance was measured at 540 nm.

PGE_2 released in the medium was measured by an enzyme immunoassay kit (Enzo Life Sciences). The detection limit was13.4 pg/ml. Absorbance was measured at 405 nm.

2.6. Statistical analysis

Each experiment was repeated at least twice. Data were analyzed by one-way ANOVA and Tukey post hoc analyses using SPSS software. The difference is significant if the *P*-value is <0.05. Indicated error bars correspond to the standard deviation (SD).

3. Results

3.1. Conditioned medium from LIPUS-stimulated osteocytes inhibits proliferation of osteoblasts

MC3T3-E1 cells were cultured in different media for 4 days. Growth curves of MC3T3-E1 cells reached their plateau within 4 days. Osteocyte-CM without LIPUS did not show any effect on the growth of cells compared to fresh medium. LIPUS-Osteocyte-CM inhibited cell proliferation from day 1, and showed significant inhibition to cell growth in day 3 and 4 compared to other two groups (Fig. 1).

3.2. Conditioned medium from LIPUS-stimulated osteocytes enhances differentiation of osteoblasts

To evaluate the effect of LIPUS-Osteocyte-CM on the differentiation of MC3T3-E1 cells, we examined the activity of ALP, a marker of osteoblastic differentiation, on day 10. LIPUS seemed to up-regulate ALP activity to some extent, but there is no significant difference compared with the control group. Ascorbic acid and β-glycerophosphate significantly increased ALP activity by two times as compared to the control group. LIPUS plus ascorbic acid

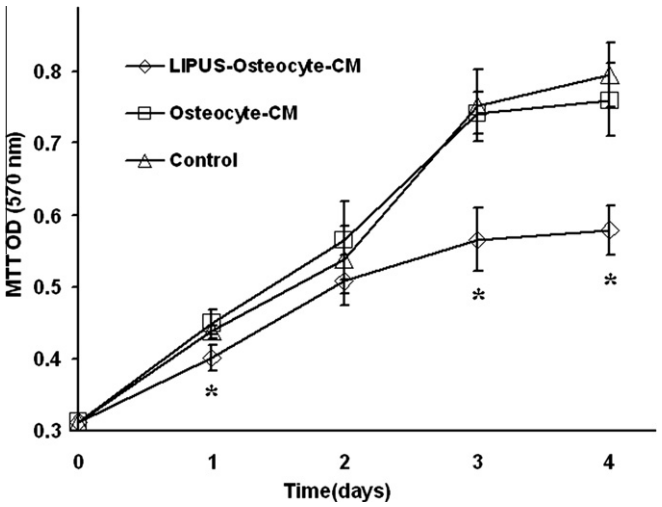

Fig. 1. Effects of LIPUS-Osteocyte-CM on proliferation of MC3T3-E1 cells. LIPUS-Osteocyte-CM, Osteocyte-CM or fresh medium were added to MC3T3-E1 cells. At 0, 1, 2, 3 and 4 days, proliferation of cells was measured through MTT method. The optical density of the dissolved formazan was read in a plate reader at 570 nm. Data are expressed as the mean ± SD ($n = 7$). Significant differences are indicated by asterisks ($P < 0.05$).