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Osteocytes exposed to far field of therapeutic ultrasound promotes osteogenic cellular activities in pre-osteoblasts through soluble factors

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ABSTRACT

Low intensity pulsed ultrasound (LIPUS) was reported to accelerate the rate of fracture healing. When LIPUS is applied to fractures transcutaneously, bone tissues at different depths are exposed to different ultrasound fields. Measurement of LIPUS shows pressure variations in near field (nearby transducer); uniform profile was found beyond it (far field). Moreover, we have reported that the therapeutic effect of LIPUS is dependent on the axial distance of ultrasound beam in rat fracture model. However, the mechanisms of how different axial distances of LIPUS influence the mechanotransduction of bone cells are not understood. To understand the cellular mechanisms underlying far field LIPUS on enhanced fracture healing in rat model, the present study investigated the effect of ultrasound axial distances on (1) osteocyte, the mechanosensor, and (2) mechanotransduction between osteocyte and pre-osteoblast (bone-forming cell) through paracrine signaling. We hypothesized that far field LIPUS could enhance the osteogenic activities of osteoblasts via paracrine factors secreted from osteocytes. The objective of this study was to investigate the effect of axial distances of LIPUS on osteocytes and osteocyte–osteoblast mechanotransduction. In this study, LIPUS (plane; 2.2 cm in diameter, 1.5 MHz sine wave, I_{SATA} = 30 mW/cm^2) was applied to osteocytes (mechanosensor) at three axial distances: 0 mm (near field), 60 mm (mid-near field) and 130 mm (far field). The conditioned medium of osteocytes (OCM) collected from these three groups were used to culture pre-osteoblasts (effector cell). In this study, (1) the direct effect of ultrasound fields on the mechanosensitivity of osteocytes; and (2) the osteogenic effect of different OCM treatments on pre-osteoblasts were assessed. The immunostaining results indicated the ultrasound beam at far field resulted in more β-catenin nuclear translocation in osteocytes than all other groups. This indicated that osteocytes could detect the acoustic differences of LIPUS at various axial distances. Furthermore, we found that the soluble factors secreted by far field LIPUS exposed osteocytes could further promote pre-osteoblasts cell migration, maturation (transition of cell proliferation into osteogenic differentiation), and matrix calcification. In summary, our results of this present study indicated that axial distance beyond near field could transmit ultrasound energy to osteocyte more efficiently. The LIPUS exposed osteocytes conveyed mechanical signals to pre-osteoblasts and regulated their osteogenic cellular activities via paracrine factors secretion. The soluble factors secreted by far field exposed osteocytes led to promotion in migration and maturation in pre-osteoblasts. This finding demonstrated the positive effects of far field LIPUS on stimulating osteocytes and promoting mechanotransduction between osteocytes and osteoblasts.

1. Introduction

A number of scientific evidence show that low-intensity pulsed ultrasound (LIPUS) treatment promotes fracture healing [1–6]. In order to further enhance the efficacy of LIPUS, scientists have been trying to modulate different important parameters of LIPUS, for examples intensity [7,8], frequency [9,10] and duty cycle [8,11]. There are, however, few reports on the parameter of axial distance.

The diffraction pattern of the ultrasound waves produced by the LIPUS transducer has two characteristic zones: near field (close to the transducer) and far field (farther from the transducer) [12]. We have reported the ultrasound beam pattern of a

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30 mW/cm^2 LIPUS beam using an ultrasound tank [13] and results indicate that the energy distribution in near field (z = 0 mm) was not uniform with maxima and minima across the beam diameter. The complexity (successive axial maxima and minima) of the ultrasound field decreases as the axial distance from transducer increases. At z = 60 mm (mid-near field), the complexity of the beam decreased and formed less maxima and minima. At z = 130 mm (far field), regular beam was formed at this axial distance [13]. When fractures were exposed to LIPUS at these mentioned axial distances, we found that far field (z = 130 mm) could further promote mineralization and mechanical properties restoration in healing callus [13]. Moreover, Reher et al. found that ex vivo calvaria bone exposed to near field (z = 0 mm, I_{SATA} = 0.1 W/cm^2 and 0.75 W/cm^2, 3 MHz) led to inconsistent results in woven bone formation. However, when the transducer was positioned farther away from the cells, consistent woven bone formation was obtained [11]. These observations indicate that the LIPUS beam region beyond near field has higher biological effect in bone cells.

Osteocytes represent 95% of bone cells in the skeleton, which are believed to function as a mechanosensor in bone owing to its characteristic morphology and intercellular networks in bone [14,15]. Osteocytes could probably transmit soluble signals directly to osteoblasts. Klein-Nulend's group had shown that secretions from pulsating fluid flow stimulated osteocytes could regulate osteoblasts through promoting differentiation [16].

In this study, we hypothesized that far field LIPUS could enhance the osteogenic activities of osteoblasts via paracrine factors secreted from osteocytes. The objective of this study was to investigate the effect of LIPUS at various axial distances on osteocytes and osteocyte–osteoblast mechanotransduction. LIPUS was applied to osteocyte cell line (MLO-Y4) at three distances: 0 mm (near field), 60 mm (mid-near field) and 130 mm (far field). The direct effect of different axial distances of LIPUS on the osteocytes was examined. The conditioned media of osteocytes (osteocyte-conditioned medium, OCM) collected from the three groups were used to culture pre-osteoblast cell line (MC3T3-E1) and the osteogenic cellular activities of the pre-osteoblasts were assessed.

2. Materials and methods

2.1. Cell culture

MLO-Y4 cells, a murine osteocyte-like cell line (generously provided by Dr. Lynda Bonewald, University of Missouri-Kansas City) [17,18], were cultured on 0.15 mg/ml rat tail type I collagen (BD Bioscience, San Jose, CA, USA) coated culture ware [18], and were maintained in α-minimal essential medium (α-MEM; Invitrogen, Carlsbad, CA, USA) containing 5% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), 5% fetal calf serum (FCS; Hyclone Laboratories, Logan, UT, USA) and 1% penicillin–streptomycin solution (Invitrogen, Carlsbad, CA, USA).

MC3T3-E1, a murine pre-osteoblast (Subclone 14, American Type Culture Center, Manassas, VA, USA), were maintained in α-MEM (without ascorbic acid supplement; Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum and 1% penicillin–streptomycin solution [19]. The MC3T3-E1 pre-osteoblast cell line is a good model for bone cell maturation studies, because it exhibits maturation sequence similar to that of pre-osteoblasts in bone tissue [20].

Cells at passages less than 25 were used to perform experiments [21–23], and were cultured in a 5% CO_2, 37 °C incubator with humidified atmosphere.

2.2. Ultrasound fields setup and treatment protocol

The ultrasound field setup was based on our established protocol [13]. Briefly, the position of the far field for an almost perfect piston transducer was determined according to the formula: $z = D2f/4c$, where z, D, f and c were near field length, transducer diameter, frequency and sound velocity in the medium, respectively [24]. The LIPUS transducer (diameter: 2.2 cm) emitted 1.5 MHz ultrasound wave propagating in rubber gel block at 1400 m/s. The computed value of z was 130 mm and defined as the far field; 0 mm was chosen as the near field; and 60 mm was chosen as the mid-near field. The consistency of the axial distances in different treatment groups was assured by standardized LIPUS treatment platforms. Each LIPUS platform consisted of six sets of LIPUS devices and rubber gel blocks, so that LIPUS could be administered to six cell culture dishes at the same time (Fig. 1). There were two types of rubber gel block: 60 mm and 130 mm in length. The LIPUS devices were tuned by the technical team of Smith & Nephew Inc. (Memphis, TN, USA) for each group to ensure the delivery of the same average power (117 mW) and intensity (I_{SATA} = 30 mW/cm^2) at the culture dish surface taking into account the attenuation of the gel block. The LIPUS devices were calibrated according to our established protocol [13]. Briefly, the LIPUS devices were calibrated by UPM-DT-1 radiation force balance (Ohmic Instruments, CO., Easton, MD).

1×10^5 of MLO-Y4 osteocyte-like cells were seeded onto culture dish (35-mm Falcon; Becton Dickinson and Company, Franklin Lakes, NJ, USA). Until 80% confluence (at day 2), medium was removed from wells. The wells were washed gently three times with PBS, so that no serum remained. Afterward, 2 ml plain α-MEM medium without any serum and antibiotics [25] was added to each well. A thin layer of ultrasound coupling gel was applied between the transducer and bottom of culture well. LIPUS either at 0 mm, 60 mm or 130 mm was applied to the MLO-Y4 cells for 20 min at 37 °C. The chance of forming standing waves in the culture wells was reduced by regulating the volume of culture medium [26]. The volume of culture medium in each well was 2 ml. The measured depth of the 2 ml-culture medium in the well was 2.1 mm, which was not a multiple of half-wavelength of the 1.5 MHz ultrasound (the calculated wavelength in medium was 1 mm). As the formation of standing waves due to ultrasound reflection depends on whether the height of culture medium in the well is an integer number of half-wavelengths of the ultrasound frequency. The probability of forming standing wave in culture well was reduced [26]. Control plates were incubated in the same incubator as the LIPUS treatment groups, but they were placed on the switched-off LIPUS treatment platform. The OCM was collected 24 h after LIPUS treatments [27]. The OCM was then centrifuged at 1500 rpm for 5 min to remove the cell debris, and the supernatant OCM was collected. Immediately after collection, 50% conditioned medium [28,29] were used to culture MC3T3-E1 pre-osteoblast cells.

The MC3T3-E1 cells were incubated with OCM for 6 and 12 h (endpoint assessment: wound healing assay) [30]; 24 and 48 h (5-Bromo-2′-deoxy-uridine; BrdU cell proliferation assay) [16]; 3 days and 6 days (alkaline phosphatase activity assay) [26]; 4 weeks (calcium nodule staining) [31]. OCM was changed every 48 h. There were five groups in the OCM treatment: Non group (plain α-MEM treatment), Con group (OCM from osteocytes without LIPUS treatment), 0 mm group (OCM from near field LIPUS treated osteocyte), 60 mm group (OCM from mid-near field LIPUS treated osteocyte) and 130 mm group (OCM from far field LIPUS treated osteocyte) (Fig. 1).

In the following individual assessments, experiments were repeated three times using the same procedures.