Low-Intensity Pulsed Ultrasound Accelerates Osteoblast Differentiation and Promotes Bone Formation in an Osteoporosis Rat Model

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Abstract
Objective: We examined the effects of low-intensity pulsed ultrasound (LIPUS) on cell differentiation, bone mineralized nodule formation and core-binding factor A1 (Cbfa1) expression in a normal human osteoblast (NHOst) cell line and bone formation in an osteoporosis animal model. Methods: NHOst cells were cultured in vitro in medium with or without LIPUS stimulation. The ultrasound stimulation frequency was 1.0 MHz at an intensity of 30 mW/cm² for 20 min. Rats were divided into a sham-operated group (Sham) and an ovariectomized group (OVX). The right femur was treated with LIPUS (Sham-LIPUS and OVX-LIPUS) and the left femur was left untreated (Sham-CON and OVX-CON). Results: LIPUS stimulation accelerated bone nodule formation and enhanced alkaline phosphatase activity. The expression levels of Cbfa1 decreased and calcification occurred earlier and more frequently in the LIPUS than in the CON groups. The wet weight of the femur increased in OVX rats with LIPUS stimulation. Morphological images showed an increase in trabecular spongiosa in the OVX-LIPUS group. Conclusion: LIPUS accelerated osteogenesis. Moreover, since LIPUS prevents bone loss, it may be a promising treatment for osteoporosis.

Introduction

Osteoporosis is the most common bone disorder found in elderly women. Its etiologies include aging, sedentary lifestyle and estrogen deficiency due to menopause, ovariectomy and hormonal therapy [1–3]. Particularly estrogen deficiency leads to rapid and marked loss of bone mineral content [4].

Low-intensity pulsed ultrasound (LIPUS) is a pressure or sound wave with the capability to transfer mechanical energy into biological tissues [5]. This acoustic energy promotes bone formation in both animals and humans with osteoporosis [6–8]. Numerous in vivo animal studies and prospective, double-blind, placebo controlled, clinical trials have shown that LIPUS is capable of accelerating and augmenting the healing of osteoporosis [9].

Accelerated healing of osteoporosis by LIPUS is attributed to the production of pressure waves, which in-
duce biochemical and molecular events at the cellular level [10]. However, the mechanotransduction pathways through which LIPUS stimulates living tissues are still not completely understood. Ott [11] showed that the ultrasound-stimulated proximal portion of the femurs of 6 patients with spinal cord injury had more bone mass than the non-stimulated one. On the contrary, Warden et al. [12] reported that 12-week LIPUS stimulation had no effect on bone mass in ovariectomized rats. Fifteen patients with spinal cord injury treated with LIPUS for 6 weeks on their calcanei continued to have decreases in bone mass similar to the non-treated patients [13].

LIPUS has been found to induce differentiation in long-bone-derived osteoblasts of adult rats in vitro [14]. It has also been reported to increase calcium accumulation in the mouse osteoblastic cell line MC3T3-E1 [15]. Even though the energy used by LIPUS is extremely low, the effects are evident. LIPUS treatment has led to increased transforming growth factor-β1 mRNA expression and aggrecan synthesis in chondrocytes [16]. Histological studies suggest that LIPUS influences all major cell types involved in bone healing, including osteoblasts, osteoclasts, chondrocytes and mesenchymal stem cells [17].

Although accelerated mineralization has been associated with increases in osteocalcin, alkaline phosphatase (ALP), vascular endothelial growth factor and matrix metalloproteinase-13 expression [18] in vitro, we found no reports concerning the effect of LIPUS on an osteoporosis model in both in vivo and in vitro conditions. Therefore, we investigated the effects of LIPUS on cell differentiation, mineralized nodule formation and core-binding factor A1 (Cbfa1) expression in a normal human osteoblast (NHOst) cell line. We also analyzed the effect of LIPUS stimulation on osteoporosis using an ovariectomized osteoporosis rat model. We hypothesize that LIPUS stimulation prevents bone loss.

**Materials and Methods**

**Cell Culture**

NHOst cells were purchased from BioWhittaker (Walkersville, Md., USA). The cells were seeded in 60-mm culture dishes (Nalge Nunc International, Roskilde, Denmark) and maintained in osteoblast proliferation medium, which contained osteoblast basal medium (OBM®, CC-3208; BioWhittaker) with 10% fetal bovine serum (CC-4102), 200 nM ascorbic acid (CC-4398) and 200 nM 1,25-dihydroxyvitamin D$_3$ (Calbiochem, La Jolla, Calif., USA), was used for the NHOst cell differentiation experiment. The cells were transferred to osteoblast differentiation medium and their concentration was adjusted to 1.2 × 10$^6$ cells/mm$^3$ using a hemocytometer (Erma, Tokyo, Japan). The cells were then seeded in 60-mm polystyrene membrane culture dishes (day 0 of culture; BD Bioscience, Bedford, Mass., USA) and cultured for 21 days at 37°C in a humidified atmosphere of 95% air and 5% CO$_2$. The medium was replaced twice weekly.

**Animals**

Twenty-seven Wistar rats (female, 16 weeks old, 246.1 ± 13.8 g) were used. The animals were housed under standard laboratory conditions on a 12-hour light/dark cycle and at a temperature of 23 ± 1°C and relative humidity of 55 ± 5%. The rats were divided into a sham-operated group (Sham, n = 12) and an ovariectomized group (OVX, n = 15). The experimental protocol was approved by the Animal Care and Use Committee of Hiroshima University.

**In vitro and in vivo LIPUS**

The NHOst cells were seeded in a polymeric membrane culture dish, the bottom of which was thin enough to transmit the ultrasound signal. The membranes of the specially constructed dishes were made of biocompatible, polyether-based thermoplastic polyurethane to facilitate the transmission of ultrasound. The method of ultrasound stimulation was similar to that described by Sena et al. [19]. In brief, the cells were divided into two groups. In the LIPUS group, the ultrasound stimulus (LIPUS; Sonic Accelerated Fracture Healing System; Teijin, Osaka, Japan) was administered through a thin silicone pad to the bottom of the culture plates. The ultrasound stimulation frequency was 1.0 MHz at an intensity of 30 mW/cm$^2$. The osteoblasts were exposed to ultrasound for 20 min on days 1, 3, 5, 7, 10, 12, 14, 18 and 21 of culture. In the control group, cells were seeded and cultured in the same manner as in the LIPUS group, but did not receive ultrasound stimulation.

One week after ovariectomy or the sham operation, rats were anesthetized with diethyl ether and the proximal portion of their right femurs was prepared to receive LIPUS (OVX-LIPUS), whereas the left femurs were left untreated (OVX-CON). The sham-operated animals were similarly grouped and treated (Sham-LIPUS and Sham-CON). The ultrasound stimulation frequency was the same as that used before. We performed the experiment for 20 min/day, 5 times/week for 5 weeks.

**Assessment of Mineralized Nodule Formation in NHOst Cells**

The NHOst cells, with or without LIPUS stimulation, were collected after 1, 3, 5, 7, 10, 12, 14, 18 and 21 days of culture. The presence of mineralized nodules was confirmed with von Kossa silver nitrate staining (Wako, Osaka, Japan) as described by Park et al. [20]. In brief, cells were rinsed with phosphate-buffered saline and fixed in 4% (w/v) paraformaldehyde in phosphate-buffered saline for 10 min. After washing with distilled water, cells were stained with 5% (w/v) silver nitrate solution and kept in the dark for 30 min. The cells were washed with distilled water to remove excess silver nitrate, and the culture plate was treated for several minutes with sodium carbonate/formaldehyde solution to develop color. Residual silver nitrate was neutralized with 5% sodium thiosulfate.
ALP Staining
The NHOS cells were rinsed three times with calcium and phosphate-free saline, fixed in 4% paraformaldehyde for 15 min at room temperature, washed three times with double-distilled water and stained with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium/ALP substrate (Amresco, Solon, Ohio, USA) for 15 min at room temperature. The cells were then washed with distilled water.

Reverse Transcription-Polymerase Chain Reaction
Total RNA of the cells was obtained using ISOGEN reagent (Nippon Gene, Tokyo, Japan). For reverse transcription, we used SuperScript II (Invitrogen, Carlsbad, Calif., USA). Sense and antisense primers for Cbfa1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, internal control) were: Cbfa1, sense 5'-CCG ATG GGA CCG TGG TTA-3' and antisense 5'-GCC CCC TAA ATC ACT GAG G-3'; GAPDH, sense 5'-CTTCCACACCATGGAGAAAGGCTG-3' and antisense 5'-ACAGTCTTTCTGAGTGCCAGTGATG-3'. The expected size of the polymerase chain reaction (PCR) products was 308 bp for Cbfa1 and 262 bp for GAPDH. Cbfa1 PCR conditions were 35 cycles for 1 min at 94°C, 1 min at 60°C and 1 min at 72°C. PCR conditions for GAPDH amplification were 24 cycles at the same temperatures and durations. The images were processed with a computer-assisted scanner and band density was analyzed using NIH Image v1.62 FPU software (National Institutes of Health, Bethesda, Md., USA).

Uterus and Femur Analysis
After 1, 3 and 5 weeks of LIPUS stimulation, the uteri and femurs were removed from rats anesthetized with excess pentobarbital. In the four treatment groups (OVX-LIPUS, OVX-CON, Sham-OVX and Sham-CON), the wet weights of uteri and both femurs were determined to investigate the effects of ovariectomy, and the femur weight/body weight ratio was calculated. LIPUS efficiency was determined by dividing femur weight in the LIPUS group by femur weight in the CON group. Radiographs were taken using micro-focus X-ray magnification equipment (micro FX-1000; FUJIFILM, Tokyo, Japan) to measure the increase in photostimulated luminescence (per mm²) using Image Gauge software (FUJIFILM).

Femur Morphology
One third of the proximal portions of the right and left femurs was embedded in paraffin, dehydrated with ethanol and demineralized for 2 weeks with 3% EDTA. Each sample was washed, dehydrated, filled with paraffin, sliced into 5-μm segments and stained with hematoxylin-eosin using the paraffin sectioning method.

Statistical Analysis
Results are expressed as means ± SD. Intergroup differences were compared by one-way analysis of variance followed by the post hoc test using StatView version 5.0 software (SAS Institute, Cary, N.C., USA), and p < 0.05 was considered statistically significant.
Morphological Changes in Femurs

Trabecular spongiosa was detected by light microscopy in the femur specimens. In the OVX group, there was a decrease in trabecular spongiosa in both right and left femurs. After 3 and 5 weeks of LIPUS stimulation, density and disruption of trabecular spongiosa were decreased in the OVX-CON group. In contrast, the density and continuity in the OVX-LIPUS group was good (fig. 7). In the Sham group, trabecular spongiosa continuity was maintained irrespective of LIPUS stimulation. Although there was no change between the OVX-LIPUS and OVX-CON groups after 1 and 3 weeks of stimulation, trabecular spongiosa tended to increase in the Sham-LIPUS group after 5 weeks of stimulation (fig. 7).

Fig. 1. LIPUS treatment accelerated bone nodule formation in cultured NHOst cells. White arrow indicates an island-like colony formation. Black arrow indicates a bone nodule. Scale bar = 100 μm.
Cbfa1 mRNA Expression

In LIPUS-stimulated femurs of the OVX group, Cbfa1 expression was slight but detectable, while it was strong in non-stimulated femurs. In the Sham group, Cbfa1 expression remained high in stimulated femurs, but was lower in non-stimulated ones (fig. 8). There was greater variability in Cbfa1 expression in the non-stimulated than the stimulated side in both groups.

Discussion

In previous studies, cells were plated in normal culture dishes and LIPUS stimulation was directed at the bottom of the dishes. However, given the acoustic dose, the functional properties of LIPUS stimulation may have changed and the shape of the LIPUS beam may have been altered. In this study, we used a novel LIPUS treatment system that enables more controlled experimental conditions.

The formation of a mineralized matrix is a definitive hallmark of osteoblastic differentiation [21, 22]. To determine whether LIPUS treatment had an effect on NHOS cell differentiation, we examined mineralized nodule formation by phase-contrast microscopy and von Kossa silver nitrate staining [23]. Our results suggested that LIPUS treatment accelerated NHOS cell differentiation by increasing mineralization.

ALP activity is an early marker of osteoblast differentiation and is critical for inducing mineralization [24]. Previous studies indicated that ALP activity is initiated during the early stages of osteoblast differentiation and continues to increase until the mineralization stage [25]. In our experiment, LIPUS stimulation induced an increase in ALP activity in early cultures (before day 10),

Fig. 2. LIPUS treatment increased bone nodule formation in cultured NHOS cells. Data are means ± SD for triplicate samples in three separate experiments. * p < 0.05 vs. the control group.

Fig. 3. LIPUS treatment increased ALP expression and mineralization. Cells were cultured in osteoblast differentiation medium for up to 7 days with or without LIPUS treatment. Cells were double stained with ALP substrate and von Kossa silver nitrate stain (mineralization). Grey = ALP-positive cells; black = mineralized deposits. Scale bar = 100 μm.

Fig. 4. The uterus wet weight was significantly lower in the OVX group compared to the Sham group. Means ± SD. * p < 0.05.
but after day 10 the increase was not significant, indicating that LIPUS affects the early stages of osteoblast differentiation by increasing ALP activity.

The uterus wet weight decreased in the OVX group (Fig. 4). It is known that the uterus atrophies are due to the lack of estrogen stimulation within 2–3 days following ovariectomy [26], indicating that the 1-week delay we allowed prior to experimentation was sufficient to remove the estrogen effect. Ovarian hypofunction was confirmed more than 2 weeks after surgery. Because there were only slight differences in the progression of bone atrophy and minimal bone mineral loss during the 5-week experiments, we could not detect a significant change in bone mineral content. Warden et al. [27] reported no difference in bone mineral content and density in a rat model of osteoporosis despite 12 weeks of LIPUS stimulation. Thus, the conversion in bone may be so low that it cannot be discriminated by X-ray. In our opinion, long-term studies investigating changes in bone mineral content and density are required to elucidate the effects of LIPUS on bone atrophy.

The bone-specific Cbfa1 gene is a runt-domain-containing transcription factor essential for osteoblastic differentiation and bone formation during embryogenesis and postnatal life [28]. We considered Cbfa1 to have two functions: (1) promotion of the initial phase of differentiation from mesenchymal stem cells to pre-osteoblasts...
Fig. 7. LIPUS treatment affected bone morphology. The morphology of bone longitudinal sections at the proximal portion of femurs was examined by hematoxylin-eosin staining. In the OVX group, trabecular spongiosa clearly diminished from 1 to 3 weeks irrespective of LIPUS stimulation (a, c/b, d). In the OVX-CON group, trabecular spongiosa disappeared or was disrupted (d, f), but there was good density and continuity in the OVX-LIPUS group (c, e). In the Sham group, the trabecular continuity was maintained. There was no effect of LIPUS after 1 and 3 weeks (a, c/b, d). After 5 weeks, the trabeculae were stronger in the Sham-LIPUS group than in the Sham-CON group (e, f). Scale bar = 2 mm.

Fig. 8. Cbfa1 mRNA expression (308 bp) was lower in the OVX-LIPUS group than in the OVX-CON group.
and (2) inhibition of pre-osteoblast differentiation to mature osteoblasts [29]. Thus, the reduced Cbfa1 mRNA expression in the OVX-LIPUS group after 3 and 5 weeks of stimulation indicated a relative acceleration of osteoblast development, which would subsequently increase the number of mature cells. This reduction in Cbfa1 expression resulted in an increase in bone matrix and mineral deposition onto the spongiosa surface strengthening the trabecular architecture, as indicated by the histology of the proximal portion of the femur in the OVX-LIPUS group. Accordingly, the wet weight of the bone was maintained resulting in an increased rate of wet weight gain by LIPUS stimulation. These results suggested that LIPUS-induced osteoblast maturation exceeded osteoblast proliferation in the OVX group.

Our study indicates that LIPUS stimulation alters Cbfa1 mRNA expression and promotes bone matrix production. Bone development and repair respond to mechanical stress, the so-called Wolff’s law [30]. Ultrasound transmits high-frequency sound waves through the body that produce mechanical stress, which in turn results in a variety of cell responses. Ultrasound has been regarded as one of the most effective mechanical stressors for bone formation. Recently, very-low-intensity ultrasound stimulation, similar to LIPUS, has been clinically applied to determine its usability to treat fractures [31]. Ultrasound vibration works directly on cells with mechanoreceptors such as bone cells, osteoblasts and chondrocytes [32, 33]. These cells respond to contraction and stimulation via stretch-activated cation channels in the cell membrane that increase intracellular calcium concentrations [34] and initiate a signaling cascade [35–37].

The effects of mechanical stress on bone remodeling using an osteoporosis model have also been reported. Oxlund et al. [38] and Flieger et al. [39] suggested that low-intensity, high-frequency vibration inhibits lower-limb bone loss. However, the rats in their studies were investigated under weight-bearing conditions, making it difficult to evaluate the independent effect of vibration. Possibly, the amplitude and velocity of the mechanical stress as well as the mechanical load act synergistically to maintain bone density. We suggest that ultrasound therapy with smaller and continuous mechanical stress, such as LIPUS, is more useful in preventing bone loss in a clinical setting.

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LIPUS Accelerates Osteoblast Differentiation

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107


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