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Low-intensity pulsed ultrasound induces apoptosis in osteoclasts: Fish scales are a suitable model for the analysis of bone metabolism by ultrasound

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ABSTRACT

Using fish scales in which osteoclasts and osteoblasts coexist on the calcified bone matrix, we examined the effects of low-intensity pulsed ultrasound (LIPUS) on both osteoclasts and osteoblasts. At 3 h of incubation after LIPUS treatment, osteoclastic markers such as tartrate-resistant acid phosphatase (TRAP) and cathepsin K mRNA expressions decreased significantly while mRNA expressions of osteoblastic markers, osteocalcin, distal-less homeobox 5, runt-related transcription factor 2a, and runt-related transcription factor 2b, increased significantly. At 6 and 18 h of incubation, however, both osteoclastic and osteoblastic marker mRNA expression did not change at least present conditions. Using GeneChip analysis of zebrafish scales treated with LIPUS, we found that cell death-related genes were upregulated with LIPUS treatment. Real-time PCR analysis indicated that the expression of apoptosis-related genes also increased significantly. To confirm the involvement of apoptosis in osteoclasts with LIPUS, osteoclasts were induced by autotransplanting scales in goldfish. Thereafter, the DNA fragmentation associated with apoptosis was detected in osteoclasts using the TUNEL (TdT-mediated dUTP nick end labeling) method. The multi-nuclei of TRAP-stained osteoclasts in the scales were labeled with TUNEL. TUNEL staining showed that the number of apoptotic osteoclasts in goldfish scales was significantly elevated by treatment with LIPUS at 3 h of incubation. Thus, we are the first to demonstrate that LIPUS directly functions to osteoclasts and to conclude that LIPUS directly causes apoptosis in osteoclasts shortly after exposure.

1. Introduction

Low-intensity pulsed ultrasound (LIPUS) provides noninvasive therapeutic treatment to accelerate fracture repair and distraction osteogenesis (see a review, Warden et al., 2000). However, most studies regarding the influence of LIPUS on bone metabolism have used osteoblast cell lines (Bandow et al., 2007; Katiyar et al., 2014; Manaka et al., 2015). Therefore, details of the direct effect of LIPUS on osteoclasts are still not fully understood because of the difficulty in handling osteoclasts. Bone consists of osteoblasts, osteoclasts, and a bone matrix, and cell-to-cell and cell-to-matrix interactions are critical for cell response to physical stress (Harter et al., 1995; Owan et al., 1997; Hoffler et al.,

Abbreviations: ALP, alkaline phosphatase; Bcl-2, B-cell lymphoma 2; CTSK, cathepsin K; Dlx5, distal less homeobox5; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; LIPUS, low-intensity pulsed ultrasound; OCN, osteocalcin; PMAIP, phorbol-12-myristate-13-acetate-induced protein 1; SAFHS, sonic accelerated fracture healing system; pNP, para-nitrophenol; TRAP, tartrate-resistant acid phosphatase; TUNEL, TdT-mediated dUTP nick end labeling; TNFRSF10A, tumor necrosis factor receptor superfamily, member 10a; Runx2a, runt-related transcription factor 2a; Runx2b, runt-related transcription factor 2b.

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Table 1
Primer sequences for real-time quantitative PCR.

Gene	Orientation	Nucleotide sequence (5′ to 3′)
TRAP	Sense	TGTCATCGTGGTTGGTCACT
	Antisense	CTCAACACCAGCTCCACTGA
CTSK	Sense	ATGATCTGGGCATGAACCAT
	Antisense	CCGAAGTGACGTATCCCAGT
OCN	Sense	TGACGTGGCCTCTATCATCA
	Antisense	TTTATAGGCGGCGATGATTC
Dlx5	Sense	GCCACGGATTCTGGCTATTA
	Antisense	TGAGCCGTAATCAGGGTAGG
Runx2a	Sense	GCTGTGCAAACCCAGAGTCC
	Antisense	CATAGGACCAGGGCGGAGAG
Runx2b	Sense	TGGCCAGTTTGAGCGCCAGT
	Antisense	AGCGCTGCCCAATGACATGC
TNFRSF10A	Sense	ATGCTCGCCCTGTGAGAAGGGCACG
	Antisense	GCCTTCTGTCGGGTAAGAGGGGCGC
PMAIP1	Sense	GGCGAAGAAAGAGCAAACCGCTG
	Antisense	GTTCAACAGATCTCCAATGTTGCGC
GAPDH	Sense	CCCCCAATGTCTCTGTTGTG
	Antisense	ACCAGCGTCAAAGATGGATG

2006). Consequently, co-culture systems that include bone matrix and both osteoclasts and osteoblasts are required to accurately assess the effect of ultrasound stimuli on bone formation and resorption. However, few techniques for a system of culturing bone cells that include bone matrix have been developed.

Teleost scale is calcified tissue that contains osteoclasts, osteoblasts, and the bone matrix of two layers (a bony layer: a thin, well-calcified external layer; and a fibrillary layer: a thick, partially calcified layer) (Bereiter-Hahn and Zylberberg, 1993; Suzuki et al., 2000; Yoshikubo et al., 2005; Azuma et al., 2007; Suzuki et al., 2007; Ohira et al., 2007; Suzuki et al., 2008). Its bone matrix, which includes type I collagen (Zylberberg et al., 1992), bone γ-carboxyglutamic acid protein (Nishimoto et al., 1992), osteonectin (Lehane et al., 1999; Redruello et al., 2005), and hydroxyapatite (Onozato and Watabe, 1979), is similar to that of mammalian bone. In light of these findings, we recently developed an *in vitro* assay system with teleost scales (Suzuki et al., 2000; Suzuki and Hattori, 2002). With this system, we examined the effect of LIPUS on the osteoclasts and osteoblasts of zebrafish scales. To analyze their detailed mechanism, we examined mRNA expression in the scales treated by LIPUS using a GeneChip system (Affymetrix). Furthermore, using osteoclasts induced by the autotransplantation of goldfish scales, we demonstrated that LIPUS treatment significantly elevated the number of apoptotic osteoclasts.

The present study is the first report that LIPUS directly functions in osteoclasts and promotes apoptosis in osteoclasts shortly after exposure.

2. Materials and methods

2.1. Animals

Zebrafish (*Danio rerio*) and goldfish (*Carassius auratus*) were purchased from a commercial source and used to analyze mRNA expression and apoptotic cells, respectively. All experimental procedures were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals of Kanazawa University, Japan.

2.2. Ultrasound conditions

Ultrasounds were generated by the Sonic Accelerated Fracture Healing System (SAFHS) 4000J (Teijin Pharma Ltd., Tokyo, Japan) through a transducer (effective area: 3.88 cm^2) at a frequency of 1.5 MHz with a pulsed-wave mode (pulse-burst width: 0.2 s; pulse repetition frequency: 1 kHz; and intensity: 30 mW/cm^2). This apparatus is the same as Exogen's SAFHS apparatus (Exogen Inc., Piscataway, NJ, USA).

2.3. LIPUS treatments and RNA isolation

Scales were collected from zebrafish under anesthesia (ethyl 3-aminobenzoate, methanesulfonic acid salt; Sigma-Aldrich, Inc., St. Louis, MO, USA) and then treated with LIPUS. After LIPUS treatment (20 min), the scales were incubated at 15 °C for 3, 6, and 18 h with Leibovitz's L-15 medium (Invitrogen, Grand Island, NY, USA) and then frozen at −80 °C for mRNA analysis. Scales not treated with LIPUS were incubated under the same conditions as the experimental group and compared with LIPUS-treated scales. Before incubation (0 h), the scales were also frozen at −80 °C and compared with incubated scales. Total RNAs were prepared from zebrafish scales using a total RNA isolation kit for fibrous tissue (Qiagen GmbH, Hilden, Germany) and treated with DNase I (RNase-Free DNase kit, Qiagen) for 15 min at room temperature to remove residual genomic DNA.

2.4. Real-time quantitative PCR assay

Real-time quantitative PCR (qPCR) assay was performed on a Real-time PCR system (Mx3000P, Stratagene Japan, Tokyo) using SYBR Premix Ex Taq (Takara Bio, Shiga, Japan) in accordance with the manufacturer's protocols. Reverse transcriptase reaction (Omniscript Reverse Transcriptase, Qiagen) was carried out with DNase-treated total RNA using an oligo (dT)16 primer. Real-time qPCR assay was performed using the specific primers listed in Table 1. Each mRNA expression level was normalized to the mRNA expression level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

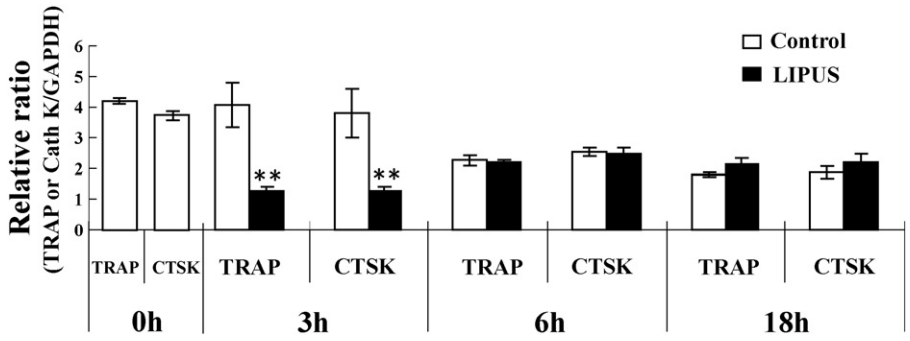

Fig. 1. The effects of LIPUS on osteoclastic markers such as TRAP (tartrate-resistant acid phosphatase) and CTSK (cathepsin K) before incubation (0) and after 3, 6, and 18 h of incubation. ** indicates statistically a significant difference at $p < 0.01$ from the values in the control scales of zebrafish.