INTRODUCTION

It is well-recognized that low intensity pulsed ultrasound (LIPUS) accelerates fracture healing, however, the mechanism underlying the ultrasound effect is not yet well understood. There have been numerous reports showing LIPUS treatment stimulating differentiation of a variety of cells such as bone marrow stromal cells, mesenchymal stem cells, chondrocytes and osteoblasts in vitro. However, there have been no reports investigating the effects of LIPUS on the cells derived from the actual human fracture site.

Haematoma occurring at a fracture site is known to play an important role in fracture healing. Recently, we discovered, for the first time, progenitor cells exist in human fracture haematoma and demonstrated those cells have the capacity for multilineage mesenchymal differentiation including osteogenic, chondrogenic, and adipogenic differentiation1.

The purpose of this study is to investigate the effect of LIPUS on human fracture haematoma-derived progenitor cells. We hypothesized that the osteogenic activity of human fracture haematoma-derived progenitor cells would be increased by LIPUS treatment.

METHODS

Patient characteristics: Fracture haematomas were obtained from 8 consecutive patients with a mean age of 24.6 years (16 to 41) during osteosynthesis, a mean of 6 days (2 to 10) after fracture. The fracture sites involved were tibia (5 patients), clavicle (1 patient) and fibula (2 patients). The project was approved by the Ethics of Human Experiments at the Faculty of Medicine, Kobe University.

Isolation and culture of human fracture haematoma-derived progenitor cells: Haematoma which had formed fibrin-clots, was removed carefully from the fracture site. Specimens were minced with a scalpel into small pieces with the original medium, α-MEM containing 10% FBS and antibiotics on 100 mm culture dish. The cultures were incubated at 37°C with 5% humidified CO2. Seven days after initial incubation, the dish was washed with PBS to remove nonviable cells and debris. Approximately two to three weeks later, the adherent cells were harvested with trypsin-EDTA and passaged for further expansion. Cells that had undergone one to three passages were used in the following assays.

Low intensity pulsed ultrasound treatment: We used a LIPUS exposure device (TEJIN PHARMA LIMITED, Tokyo, Japan) which is adapted to the 6-well culture plate. This device produces a wave equal to the conditions of sonic accelerated fracture healing system (SAFHS, TEJIN) for clinical use. 5 × 10⁴ cells per well were seeded into a 6-well plate and cultured until they reached subconfluence for all experiments except cell counting. The medium was replaced with a fresh osteogenic medium consisting of the original medium, 10 mM β-glycerophosphate, and 50 μg/ml ascorbic acid. The culture plate was placed on the ultrasound transducer with a thin layer of water to maintain contact. LIPUS was given through the bottom of the culture plates for 20 minutes daily at 37°C on the indicated days. Cells without LIPUS treatment acted as controls.

Cell count: 5 × 10⁴ cells per well were seeded into a 6-well plate and stationary cultured for 2 days. LIPUS was applied for additional 2, 4 and 7 days. An original medium was used for this experiment. Cells were detached with trypsin-EDTA. The number of cells was counted twice using a hemacytometer and the average was calculated. Cell viability was >99% by the trypan blue dye exclusion technique.

Alkaline phosphatase (ALP) activity assay: LIPUS was applied for 2, 4, 7 and 14 days. ALP activities of extracted samples were assayed by measuring the release of p-nitrophenol from p-nitrophenyl phosphate as substrate.

Osteocalcin (OCN) secretion assay: LIPUS was applied for 2, 4, 7, 14 and 28 days. After removing the medium, 2 ml original medium plus 1 μM 1,25(OH)₂ vitamin D₃ was added and incubated at 37°C for 24 hours. The medium in each well was collected and OCN secretion was quantified using Gla-OC Competitive enzyme immunoassay (EIA) Kit (TaKaRa, Shiga, Japan).

Total RNA extraction and RT-PCR: LIPUS was applied for 2, 4, 7, 14 and 28 days. At one hour after the LIPUS treatment of each group, expression of osteoblast-related genes, runt-related gene 2 (Runx2), Osterix (OSX), parathyroid hormone receptor (PTH-R), osteopontin (OPN), and bone sialoprotein (BSP) was measured by RT-PCR. Total RNA was extracted from the cell layer and reverse-transcribed to synthesize cDNA. PCR was carried out under specific conditions for the individual primers. Band intensities were semi-quantified using Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) as an internal control.

Mineralization assay: LIPUS was applied for 28 days. The cultures were stained with 1% alizarin red S and the staining was released from the cell matrix by incubation in 10% ethylenediamine chloride. The amount of dye released was quantified by spectrophotometer at 562 nm.

Statistical analysis: To assess differences between LIPUS-treated cells and the control, signed Wilcoxon's rank sum test was performed. A value of p < 0.05 was considered to be statistically significant.

RESULTS

Proliferation: There was no significant difference in the total number of cells between LIPUS-treated cells and the control at 2, 4, and 7 days.

ALP activity: ALP activity in LIPUS-treated cells was significantly higher than in the control at 2, 4, 7 and 14 days (p < 0.05). Maximum fold change was 1.4 at day 14.

OCN secretion: OCN secretion of LIPUS-treated cells was significantly higher than the control at 4, 7, 14 and 28 days (p < 0.05), but not at 2 days. The maximum fold change was 2.2 at day 28.

Gene expression of osteoblast-related genes: The gene expression of Runx2, OSX, PTH-R, OPN, and BSP in LIPUS-treated cells was significantly higher than the control at 2, 4, 7, 14 and 28 days (p < 0.05). The maximum fold change was 2.3 for Runx2, 4.8 for OSX, 4.3 for PTH-R, 4.4 for OPN, and 6.4 for BSP.

Mineralization: Alizarin red S staining of LIPUS-treated cells was significantly higher than the control at 28 days (p < 0.05). The fold change was 8.4.

DISCUSSION

These results indicate that osteogenic differentiation of human fracture haematoma-derived progenitor cells is promoted by LIPUS treatment whereas cell proliferation is not affected.

The mechanism underlying the ultrasound effect is not yet well understood. Runx2 plays a crucial role in the early determination stage of the osteoblast lineage, whereas OSX regulates the later stage of osteoblast differentiation and bone formation. We revealed one possible mechanism for osteogenic differentiation of human fracture haematoma-derived progenitor cells was promoted by high expressions of Runx2 and OSX after LIPUS treatment.

This is the first report demonstrating the biological response of cells derived from the actual human fracture site to LIPUS treatment. This study provides significant evidence for the clinical application of LIPUS for fracture treatment.

REFERENCE