INTRODUCTION

Although fracture healing often occurs without complications, a significant proportion (5 to 10%) of fractures fail to heal and result in delayed union or persistent nonunion [1]. To enhance the fracture healing, a variety of treatment techniques have been developed. It is well-recognized that low-intensity pulsed ultrasound (LIPUS) accelerates healing of fractures and non-unions [2]. Bone morphogenetic protein-7 (BMP-7) has also reported to promote bone formation in non-unions and spinal fusion [3]. It is possible that combination of the two therapies may provide better results than with either treatment alone.

Hematoma occurring at a fracture site is known to play an important role in fracture healing. Recently, we demonstrated that mesenchymal progenitor cells (MPCs) with multilineage differentiation potential exist in human fracture hematoma, indicating its critical role in the process of fracture healing [4]. Previously, we demonstrated that the in vitro osteogenic activity of human fracture hematoma-derived MPCs (HMPCs) was enhanced by LIPUS treatment. We hypothesized that the combined application of BMP-7 and LIPUS will cause an additive or synergistic effect on osteogenesis of HMPCs.

MATERIALS and METHODS

Patient characteristics: This study was approved by the institutional ethical committee and informed consent was obtained from all study subjects. Fracture hematoma was obtained from 4 patients during osteosynthesis, a mean of 5.6 days (4 to 7) after fracture. The fracture sites involved were tibia (1 patient) and fibula (3 patients).

Isolation and culture of HMPCs: Hematoma which had formed fibrin clots was removed from the fracture site. Specimens were minced, and digested with Collagenase II. Isolated cells were cultured in the growth medium, α-MEM containing 10% fetal bovine serum and antibiotics. At subconfluence, the adherent cells were harvested with trypsin-EDTA and passaged for further expansion. Cells at passage 2 were used in the following assays. 3 x 10^5 cells per well were seeded into a 6-well plate. After 4 days, the medium was replaced with a fresh culture medium. Four experimental groups were studied:

1: Control (growth medium, no ultrasound)
2: BMP-7 alone (osteogenic medium)
3: Ultrasound alone (growth medium)
4: BMP-7 (osteogenic medium) + Ultrasound

The osteogenic medium consisted of the growth medium with 10 mM β-glycerophosphate, 50 μg/ml ascorbic acid, and 1 ng/ml rhBMP-7.

Ultrasound stimulation: We used a LIPUS exposure device (Teijin Pharma Ltd., Tokyo, Japan). This device produces a wave equal to the wave conditions of sonic accelerated fracture healing system (SAFHS) for clinical use. The 6-well culture plate was placed on the ultrasound transducer with a thin layer of water to maintain contact. LIPUS (30 mW/cm² intensity at 1.5 MHz) was given through the bottom of the culture plates for 20 minutes daily at 37°C for 14 days.

Alkaline phosphatase (ALP) activity assay: ALP activities of extracted samples at day 14 were assayed by measuring the release of p-nitrophenol from p-nitrophenyl phosphate as substrate.

Real-time Polymerase Chain Reaction (PCR) Analysis: Expression of osteoblast-related genes, runt-related gene 2 (Runx2), ALP, osteopontin (OPN), and GAPDH was measured by real-time PCR. The level of each target gene was normalized to GAPDH levels and expressed relative to the day-0 control culture levels (ΔΔCt methods).

Mineralization assay: After 14 days, the cultures were stained with 1% alizarin red S at pH 4.0 for 5 minutes, washed with water, and dried.

RESULTS

ALP activity: After 14-day culture, ALP activity in group 4 was highest, and significantly higher than in group 2 (p < 0.05).

Gene expression of osteoblast-related genes: The gene expression of Runx2, ALP, and OPN in group 4 was higher than in other groups at day 14 (p < 0.05). The fold change in group 4 at day 14 was 4.8 for Runx2, 6.2 for ALP, and 2.7 for OPN, respectively.

Mineralization: The intensity of Alizarin red S staining of HMPCs in group 4 was significantly higher than other groups at day 14 (p < 0.05).

DISCUSSION

The novel finding of this study is that LIPUS treatment enhanced BMP-7-induced bone formation of HMPCs in vitro. Our results clearly showed that combined treatment with LIPUS and BMP-7 had additive effect on osteoblast-related gene expression, ALP activity, and Alizarin Red S staining. The mechanism by which LIPUS enhances BMP-7-induced bone formation is not known. Both LIPUS and BMP-7 are known to act on different phases of osteogenesis, and together they may act in a sequential manner on selective phases, thus leading to enhanced osteogenesis.

This is the first report demonstrating the biological response of cells derived from the actual human fracture site to combined treatment of LIPUS and BMP-7. This study provides significant evidence for the clinical application of combined application of LIPUS and BMP-7 for fracture treatment.


Effect of Low-Intensity Pulsed Ultrasound and BMP-7 on Osteogenic Differentiation of Human Fracture Hematoma-Derived Cells in Vitro

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