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Research Paper
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Low-intensity pulsed ultrasound enhances bone morphogenetic protein expression of human mandibular fracture haematoma-derived cells

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Abstract. We previously demonstrated that human mandibular fracture haematoma-derived cells (MHCs) play an important role in mandibular fracture healing and that low-intensity pulsed ultrasound (LIPUS) accelerates this effect by stimulating various osteogenic cytokines. In the present study, we investigated how LIPUS affects the expression of bone morphogenetic proteins (BMPs), which are also known to have the ability to induce bone formation. MHCs were isolated from human mandibular fracture haematomas and the cells were divided into two groups: a LIPUS (+) group and a LIPUS (−) group, both of which were cultured in osteogenic medium. LIPUS was applied to the LIPUS (+) group 20 min a day for 4, 8, 14, and 20 days (1.5 MHz, 30 mW/cm^2). Real-time PCR and immunofluorescence studies were carried out to determine the expression of BMP-2, 4, and 7. Compared to the LIPUS (−) group, gene expression levels were significantly increased in the LIPUS (+) group for BMP-2 on day 20 (67.38 ± 26.59 vs. 11.52 ± 3.42, $P < 0.001$), for BMP-4 on days 14 (45.12 ± 11.06 vs. 9.20 ± 2.88, $P = 0.045$) and 20 (40.96 ± 24.81 vs. 3.22 ± 1.53, $P = 0.035$), and for BMP-7 on day 8 (48.11 ± 35.36 vs. 7.03 ± 3.96, $P = 0.034$). These findings suggest that BMP-2, 4, and 7 may be mediated by LIPUS therapy during the bone repair process.

Key words: low-intensity pulsed ultrasound (LIPUS); bone morphogenetic protein (BMP); mandibular fracture; haematoma; osteogenesis; real-time PCR; immunofluorescence.

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Low-intensity pulsed ultrasound (LIPUS) is a safe and well-established therapeutic modality that is frequently used to accelerate fracture healing without surgical invasion. This modality is a source of mechanical energy transmitted as acoustic pressure waves into biological tissues, which subsequently evoke biochemical events that regulate fracture healing.[1] Many clinical and experimental studies have shown that LIPUS stimulates the differentiation of a variety of cells,

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including osteoblasts and bone marrow stromal cells, thus enhancing bone regeneration by upregulating various cytokines and growth factors.[2,3] However, the mechanisms by which LIPUS acts on osteoblasts and bone healing remain unclear.

Bone morphogenetic proteins (BMPs) are well-known cytokines that play important roles in osteogenesis. These cytokines were originally discovered based on their ability to induce bone formation. Of the over 20 different isoforms of BMP described to date, three members of the BMP family, BMP-2, BMP-4, and BMP-7, are thought to play important roles in the skeletal system and fracture healing.[4,5]

BMPs derived from mesenchymal cells inhibit muscle differentiation and promote the chemotactic properties that stimulate the differentiation of mesenchymal cells into osteoblasts. BMPs also affect bone remodelling via the regulation of osteoclast bone resorption.[6]

Previous experiments by our group have demonstrated that cells within mandibular fracture haematomas contribute to mandibular fracture healing and that the osteogenic activity of human mandibular fracture haematoma-derived cells (MHCs) is enhanced by LIPUS stimulation.[7]

Several in vitro investigations of animal cells suggest that LIPUS stimulation also increases BMP expression.[2,8,9] Moreover, evidence suggests that LIPUS induces osteogenic differentiation of human periodontal ligament cells by activating BMP-2 signaling.[10] However, how the LIPUS, which is mainly used to accelerate fracture healing, regulates BMP expression in humans has not been fully elucidated, especially in the case of human mandibular fractures.

We hypothesized that the gene expression of BMPs would be promoted after the application of LIPUS to the sites of mandibular fractures. This study demonstrates the effects of LIPUS on BMP expression during the osteogenesis of MHCs.

Materials and methods

Specimens of mandibular fracture haematomas were obtained from six patients during osteosynthesis, at a mean 3.7 days (range 1–9 days) after injury. These patients had a mean age of 39.7 years (range 17–71 years). In delayed cases, we scrutinized the patient's general condition prior to treatment; the delays were not due to the study protocol. The fractures involved the median mandible in three patients and the mandibular body in three patients. Subjects who had received anticoagulants, steroids, or non-steroidal anti-inflammatory drugs within the 3 months prior to injury were excluded.

This project was approved by the ethics committee of human experiments of the university faculty of medicine; informed consent was obtained from all patients.

Isolation and culture of MHCs

Fracture haematomas that had formed fibrin clots were removed manually prior to any manipulation or irrigation and were placed in sterile polypropylene containers to avoid contamination during surgery.[7] The mean wet weight of the haematomas was 1.1 g (range 0.1–4.2 g). The specimens were cut into small pieces with a scalpel in growth medium (α-Modified Minimum Essential Medium; Sigma, St. Louis, MO, USA), containing 10% heat-inactivated foetal bovine serum (Sigma), 2 mM L-glutamine (Gibco BRL, Grand Island, NY, USA), and antibiotics (penicillin G, 100 units/ml; streptomycin, 100 μg/ml). The samples were then incubated at 37 °C in 5% humidified CO_2. The culture medium was changed twice weekly. Approximately 3–4 weeks later, the adherent cells were harvested with 0.05% trypsin containing 0.02% ethylenediaminetetraacetic acid (EDTA; Wako, Osaka, Japan) and passaged into non-coated 150-cm^2 culture flasks. Cells that had undergone one to three passages were used in the assays.

LIPUS treatment

We used a LIPUS exposure device (Teijin Pharma Ltd., Tokyo, Japan) adapted to a six-well tissue cell culture plate in the in vitro experiment. The device was set at a 1.5 MHz sine wave with a pulse duration of 200 μs, repeating pulse at 1 kHz, and intensity of 30 mW/cm^2. This waveform is equal to the wave conditions of a sonic-accelerated fracture healing system (SAFHS; Teijin Pharma Ltd.). A total of 5×10^4 MHCs per well were seeded into a six-well plate until they reached subconfluence. The medium was replaced with fresh osteogenic medium consisting of the growth medium, 10 mM β-glycerophosphate (Sigma), and 50 μg/ml of ascorbic acid (Wako). The culture plate was then placed on the ultrasound transducer with a thin layer of water to maintain contact. LIPUS was applied through the bottom of each culture plate for 20 min daily at 37 °C for 4, 8, 14, and 20 days. Cells treated without LIPUS stimulation served as the control group.

Total RNA extraction and real-time polymerase chain reaction (PCR)

In order to detect the expression levels of the BMPs, total RNA was extracted from the harvested cells on days 0, 4, 8, 14, and 20 after the LIPUS or sham treatment using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. Total RNA was reverse-transcribed into single-stranded cDNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). The expression levels of BMP-2, 4, and 7 were measured using real-time PCR. The real-time PCR analysis was performed in duplicate using an ABI PRISM 7700 Sequence Detection System and SYBR Green reagents (Applied Biosystems) following the recommended protocols. The housekeeping gene, glyceraldehyde-3-phosphate (GAPDH), was used to monitor RNA loading. The primers used for amplification are shown in Table 1. The expression levels of all genes were normalized to the GAPDH levels and expressed relative to the day 0 control culture level (ΔΔCT method; Applied Biosystems). The mRNA expression was then compared between the LIPUS (+) and LIPUS (−) groups. The expression levels were expressed as the relative fold increase compared to the day 0 control level.

Table 1. Details of the primers used for real-time PCR amplification.

Gene	Primer sequences (5′ to 3′)
BMP-2	F: 5′ GGAACGGACATTCGGTCCT 3′
	R: 5′ GGAAGCAGCAACGCTAGAAG 3′
BMP-4	F: 5′ TCACTGCAACCGTTCAGAGGTC 3′
	R: 5′ CCAATCTTGAACAAACTTGCTGGA 3′
BMP-7	F: 5′ ACCAGAGGCAGGCCTGTAAGA 3′
	R: 5′ CTCACAGTAGTAGGCGGCGTAG 3′
GAPDH	F: 5′ CCACCCATGGCAAATTCCATGG 3′
	R: 5′ TCTAGACGGCAGGTCAGGTCCA 3′

BMP, bone morphogenetic protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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