Osteogenic activity of human fracture haematoma-derived progenitor cells is stimulated by low-intensity pulsed ultrasound in vitro

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The haematoma occurring at the site of a fracture is known to play an important role in bone healing. We have recently shown the presence of progenitor cells in human fracture haematoma and demonstrated that they have the capacity for multilineage mesenchymal differentiation. There have been many studies which have shown that low-intensity pulsed ultrasound (LIPUS) stimulates the differentiation of a variety of cells, but none has investigated the effects of LIPUS on cells derived from human fracture tissue including human fracture haematoma-derived progenitor cells (HCs). In this in vitro study, we investigated the effects of LIPUS on the osteogenic activity of HCs. Alkaline phosphatase activity, osteocalcin secretion, the expression of osteoblast-related genes and the mineralisation of HCs were shown to be significantly higher when LIPUS had been applied but without a change in the proliferation of the HCs.

These findings provide evidence in favour of the use of LIPUS in the treatment of fractures.

In the process of the healing of fractures a haematoma is initially formed and is known to have an important role in healing. Mizuno et al1 reported that the haematoma had an inherent osteogenic potential which contributed significantly to healing. Grundnes and Reikeras2 found that removal of an organised haematoma some days after fracture impaired healing. We have recently reported that progenitor cells are present in human fracture haematoma and have the capacity for multilineage mesenchymal differentiation.3

Innumerable fractures occur annually as a result of human activity, morbidity and bone fragility.4,5 Low-intensity pulsed ultrasound (LIPUS) has been used to manage refractory fracture healing6 and its effectiveness has been confirmed.6-9 However, the mechanism underlying the effect of ultrasound is not well understood. LIPUS has been shown to stimulate the differentiation of a variety of cells including bone-marrow stromal cells, mesenchymal stem cells, chondrocytes and osteoblasts in vitro.10-22 However, there have been no reports of its effect on cells derived from human fracture tissue including haematoma-derived progenitor cells (HCs).

We have investigated the effect of LIPUS on HCs derived from human fracture haematoma hypothesising that the osteogenic activity of these HCs would be increased by LIPUS.

Patients and Methods
Specimens of fracture haematomas were obtained from eight consecutive patients with a mean age of 24.6 years (16 to 41) during osteosynthesis at a mean of six days (2 to 10) after injury. The fracture involved theibia in five patients, the clavicle in one and the fibula in two. Patients taking anticoagulants, steroids or non-steroidal anti-inflammatory drugs in the three months before the injury were excluded. The project was approved by the Ethics of Human Experiments at the Faculty of Medicine, Kobe University and informed consent was obtained from all the patients.

Isolation and culture of HCs. Haematoma which had formed fibrin clots was removed manually before any manipulation or irrigation and placed in sterile polypropylene containers in order to avoid contamination during the operation. The mean wet weight of the haematoma obtained was 1.1 g (0.4 to 2.0). Specimens were cut into small pieces with a scalpel in original medium, α-Minimum Essential Medium (Sigma, St. Louis, Missouri) containing 10% heat-inactivated fetal bovine serum (Sigma), 2mM L-glutamine (Gibco BRL, Grand Island, New York) and antibiotics (penicillin G, 100 units/ml and streptomycin 100 μg/ml) on 100 mm culture dishes. The cultures were incubated at 37°C in 5% humidified CO2. Seven days after the initial incubation, the dishes were...
washed with phosphate-buffered saline (PBS; Wako, Osaka, Japan) to remove non-viable cells and debris and thereafter the culture medium was changed twice weekly. Approximately two to three weeks later, the adherent cells were harvested with 0.05% trypsin-0.02% ethylene diamine tetra-acetic acid (EDTA, Wako) and passaged into non-coated 75 cm² culture flasks with a density of approximately 2 × 10⁵ cells/75 cm² per flask for further expansion. Cells which had undergone one to three passages were used in the assays.

**Low-intensity pulsed ultrasound treatment.** We used a LIPUS exposure device (Teijin Pharma Ltd, Tokyo, Japan) which was adapted to a six-well tissue-cell culture plate in the in vitro experiment. It was set at a 1.5 MHz sine wave with a pulse duration of 200 μs, a repeating pulse at 1 kHz and an intensity of 30 mW/cm². This waveform was equal to the wave conditions of a sonic-accelerated fracture healing system (SAFHS, Teijin Pharma Ltd). Briefly, 5 × 10⁴ HCs per well were seeded into a six-well plate until they reached subconfluence. The medium was replaced with fresh osteogenic medium consisting of the original 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 the culture plates for 20 minutes daily at 37°C for two, four, seven, 14 and 28 days. Cells without LIPUS stimulation acted as a control group. The cells were subjected to the following biochemical assays and reverse transcription polymerase chain reaction (RT-PCR) analysis after the treatment. Three wells were used for each treatment group and the mean was calculated for the eight patients.

**Cell count.** A total of 5 × 10⁴ HCs per well was seeded into a six-well plate and stationary cultured for two days. LIPUS stimulation was applied according to the described protocol for two, four or seven days. The original medium was used for all groups. HCs were detached using 0.05% trypsin-0.02% EDTA (Wako). The number of HCs was counted twice using a Hemacytometer (Bio-Rad Laboratories Japan, Tokyo, Japan) and the mean was calculated. Cell viability was found to be > 99% by the Trypan Blue dye (Gibco BRL) exclusion technique.

**Alkaline phosphatase (ALP) activity assay.** LIPUS stimulation was applied for two, four, seven and 14 days. At 24 hours after the LIPUS treatment of each group, the ALP activity of the extracted samples was assayed. The cell layer from each well was washed twice with PBS, sonicated with a Microson Ultrasonic Cell Diaruptor XL2000 (Misonix, Farmingdale, New York) and stored at -20°C until the assay was undertaken. ALP activity was assayed as the release of p-nitrophenol from p-nitrophenylphosphate, pH 9.8, and the p-nitrophenol release was monitored by optical density at 405 nm using the SenoLyte pNPP Alkaline Phosphatase Assay Kit (AnaSpec Corporate Headquarters, San Jose, California). The protein concentration in the sonicate was measured by the BCA Protein Assay Kit (Pierce Chemical Co, Rockford, Illinois). The results were expressed as p-nitrophenol produced in nmol/min/mg of protein.

**Osteocalcin (OCN) secretion assay.** LIPUS stimulation was applied for two, four, seven, 14 and 28 days. After removing the medium, 2 ml of original medium and 10⁻⁸ mol/l 1.25(OH)₂ vitamin D₃ were added and incubated at 37°C for 24 hours. The medium in each well was collected and the OCN secretion was quantified using the BCA Protein Assay Kit (Pierce Chemical Co, Rockford, Illinois). The results were expressed as p-nitrophenol produced in nmol/min/mg of protein.

### Table I. Details of the primers used for amplification

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences (5′ to 3′) (sense/antisense)</th>
<th>Product size (bp)</th>
<th>Annealing temperature (°C)</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>CCACCCATGGCAAAATCCATGGCA TCTAGACGGCAGTCCACCC</td>
<td>593</td>
<td>55</td>
<td>25</td>
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<tr>
<td>BSP</td>
<td>ATTTCAGGTTAGGGCCAGTAG ACATTCTTCTCCTCAGGGCTT</td>
<td>447</td>
<td>57</td>
<td>35</td>
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<tr>
<td>OPN</td>
<td>GGCTAACCTGGCCACCCTC TCATTGGTCTCATCTATGTGCCG</td>
<td>640</td>
<td>60</td>
<td>35</td>
</tr>
<tr>
<td>OSX</td>
<td>AACCCTGGCTGGCCACCTA TCGCTAGCTACGTCCAAACAG</td>
<td>457</td>
<td>60</td>
<td>30</td>
</tr>
<tr>
<td>Runx2</td>
<td>AGTTCCAAAGCATTTTATGC TCAATATGGTCGCGAAACAG</td>
<td>421</td>
<td>57</td>
<td>34</td>
</tr>
<tr>
<td>PTH-R</td>
<td>AGGCCAGGCACTATAGGAA GCTCCGTTACAGGCTCTCAT</td>
<td>374</td>
<td>60</td>
<td>35</td>
</tr>
</tbody>
</table>

* GAPDH, glyceraldehyde-3-phosphate dehydrogenase; BSP, bone sialoprotein; OPN, osteopontin; OSX, osterix; Runx2, runt-related; PTH-R, parathyroid hormone receptor
Total RNA extraction and RT-PCR analysis. LIPUS stimulation was applied for two, four, seven, 14 and 28 days. At one hour after the LIPUS treatment of each group, expression of osteoblast-related genes, bone sialoprotein (BSP), osteopontin (OPN), runt-related gene 2 (Runx2), Osterix (OSX) and parathyroid hormone receptor (PTH-R) were also measured by RT-PCR. In all RT-PCR assays, the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was analysed to monitor RNA loading. Dedicated primers were used for amplification as shown in Table I. The products were visualised by the luminescence of ethidium bromide by ultraviolet rays after electrophoresis in 2% agarose gel. The expression levels of the genes were quantified using a densitometric program (Scion Image for Windows; Scion Corporation, Frederick, Maryland). After normalising by the band intensity of GAPDH, the percentage changes of the genes were determined.

Mineralisation assay. The cells were treated with LIPUS stimulation for 20 minutes daily for four weeks in a six-well plate and then fixed for one hour at room temperature in 95% ethanol. The plate was stained with 1% Alizarin Red S (Hartman Leddon, Philadelphia, Pennsylvania) at pH 4.0 (Sigma) for five minutes, washed with water and dried. Alizarin Red S staining was released from the cell matrix by incubation in 10% ethylpyridinium chloride for 15 minutes with the development of red staining indicating a positive result. The amount of dye released was quantified by spectrophotometry at 562 nm.

Results

Proliferation. There was no significant difference (Wilcoxon’s rank-sum test) in the total number of cells in the LIPUS and control groups at two, four and seven days of treatments (Fig. 1).
**ALP activity.** In LIPUS-treated cells the ALP activity was significantly higher than that in the control group at two, four, seven and 14 days after treatment (p < 0.05, Wilcoxon’s rank-sum test). The percentage change in ALP activity.
activity after 14 days of treatment was the highest in all eight specimens (Fig. 2).

**OCN secretion.** In LIPUS-treated cells OCN secretion was significantly higher than that in the control group at four, seven, 14 and 28 days (p < 0.05, Wilcoxon’s rank-sum test). The percentage change in OCN secretion after the 28-day treatment was the highest in all eight specimens (Fig. 3).

**Gene expression.** Osteogenic activities were up-regulated by LIPUS treatment and were further confirmed by RT-PCR analysis showing the expression of BSP, OPN, Runx2, OSX and PTH-R at two, four, seven, 14 and 28 days. The expressions after LIPUS treatment were significantly higher than those in the control group at each interval for all genes (p < 0.05, Wilcoxon’s rank-sum test) (Fig. 4).

**Mineralisation.** Alizarin Red S staining of LIPUS-treated cells was significantly higher than in the control group after four weeks of treatment group (p < 0.05, Wilcoxon’s rank-sum test) (Fig. 5).

**Discussion**

Our study has shown that the osteogenic activity of HCs is increased by LIPUS treatment.

The ALP activity, the OCN secretion, the expression of osteoblast-related genes and the mineralisation of HCs were significantly higher in the LIPUS treatment group than in the control group indicating that osteogenic differentiation of HCs is promoted by LIPUS. The effect of LIPUS on fracture healing has been described in clinical and laboratory studies. One *in vitro* study suggested that it had a stimulatory effect on calvarian osteoblasts in rats with increased ALP activity. Some have shown an increase in the transcription of OCN mRNA in osteocytes after mechanical stimulation. Additionally, LIPUS has been shown to increase mRNA levels in the bone-matrix proteins ALP and OCN in a rat osteosarcoma cell line and a mouse osteoblastic cell line. LIPUS has also been shown to increase mRNA levels for OP and Runx2 in CD-
1 mice osteoblasts. There have been numerous studies on the stimulatory effect of LIPUS in a variety of cells in vitro, but the cells in these reports were not derived from a human fracture site and it is unclear whether the findings can be applied to man. Our work on cells derived from human fracture tissue confirms that the osteogenic differentiation of HCs is promoted by LIPUS.

The mechanism underlying the ultrasound effect is not well understood. Azuma et al studied the relationship between the timing of partial LIPUS treatment and the efficiency of accelerating the action of healing. They reported that LIPUS accelerated fracture healing and that, at an inflammatory phase, macrophages contained a stretch-sensitive potassium channel, the activity of which was modified by mechanical stress. This may be one mechanism by which LIPUS affects fracture healing. Runx2, a member of the runt family of transcription factors, has a crucial role in the early determination stage of the osteoblast lineage, whereas OSX, a zinc-finger-containing transcription factor encoded by SP7, regulates the later stage of osteoblast differentiation and bone formation. In our study, we have shown that one mechanism for osteogenic differentiation of HCs was promoted by the high expressions of Runx2 and OSX after LIPUS treatment.

It is widely agreed that the presence of growth factors, specifically transforming growth factor beta, insulin-like growth factor, platelet-derived growth factor, acidic fibroblast growth factor, and basic fibroblast growth factor, within the fracture haematoma is a prerequisite for the regulation of the process which occurs during fracture healing. Some studies have shown that mesenchymal stem cells are differentiated into osteoblasts and/or chondrocytes under the influence of several growth factors. Recently, we described one possible mechanism for fracture healing at all phases. Further studies are needed to discover whether growth factors released from HCs are increased by LIPUS treatment.

There was no significant difference in the total number of cells produced after LIPUS treatment compared with that of the control group indicating that LIPUS did not affect cell proliferation. Some reports indicated that LIPUS affected the proliferation of osteoblasts in rat calvaria, and in CD-1 mice and in human periosteal cells. By contrast, others have indicated that LIPUS affects mainly cell differentiation rather than cell proliferation in human mesenchymal stem cells, rabbit chondrocytes, rat femoral fracture cells, and rat fracture callus cells. In our study, we investigated progenitor cells derived from human fracture haematoma which may indicate that in HCs LIPUS affects cell differentiation more than proliferation.

In this in vitro study, we have found that LIPUS increased the osteogenic activity of HCs and have demonstrated that progenitor cells in actual human fracture haematoma were affected by LIPUS treatment. Our study provides further evidence in favour of the clinical application of LIPUS in the treatment of fractures.

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References


T. HASEGAWA, M. MIWA, Y. SAKAI, T. NIIKURA, M. KUROSAKA, T. KOMORI
OSTEOGENIC ACTIVITY OF HUMAN FRACTURE HAEMATOMA-DERIVED PROGENITOR CELLS IS STIMULATED BY ULTRASOUND


