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Research Paper

Trauma

The osteogenic activity of human mandibular fracture haematoma-derived cells is stimulated by low-intensity pulsed ultrasound in vitro

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Abstract. Low intensity pulsed ultrasound (LIPUS) stimulation is a clinically established treatment method used to accelerate long bone fracture healing; however, this method is currently not applied to mandibular fractures. In this study, we investigated the effects of LIPUS on human mandibular fracture haematoma-derived cells (MHCs) in order to explore the possibility of applying LIPUS treatment to mandibular fractures. MHCs were isolated from five patients. The cells were divided into two groups: (1) LIPUS (+) group: MHCs cultured in osteogenic medium with LIPUS treatment; and (2) LIPUS (−) group: MHCs cultured in osteogenic medium without LIPUS treatment. The osteogenic differentiation potential and proliferation of the MHCs were compared between the two groups. The waveform used was equal to the wave conditions of a clinical fracture healing system. The gene expression levels of ALP, OC, Runx2, OSX, OPN, and PTH-R1 and mineralization were increased in the LIPUS (+) group compared to the LIPUS (−) group. There were no significant differences in cell proliferation between the two groups. These findings demonstrate the significant effects of LIPUS on the osteogenic differentiation of MHCs. This study provides significant evidence for the potential usefulness of the clinical application of LIPUS to accelerate mandibular fracture healing.

Key words: mandibular fracture; haematoma; low intensity pulsed ultrasound (LIPUS); osteogenic activity.

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Mandibular fractures are the second most common fracture of the facial bones. Thousands of mandibular fractures occur annually in the USA.[1] It is well known that the mandible is an intramembranous bone in embryology, and that the mandibular fracture healing process involves intramembranous ossification without cartilage formation.[2–4] Following intramembranous bone fractures, mesenchymal cells from the periosteum differentiate directly into osteoblasts; these form osteoid tissue,

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which is subsequently mineralized.[5] Of interest, several investigators have reported the presence of cartilage formation, namely endochondral ossification, during the healing of mandibular fractures.[5,6] Though it is not clear whether or not cartilage formation occurs, mandibular healing is mainly intramembranous ossification.

It is known that the fracture haematoma plays an important role in fracture healing. Mizuno et al. have reported that the fracture haematoma has inherent osteogenic potential, which contributes significantly to the healing of long bone fractures.[7] Grundnes and Reikeras have reported that the removal of an organized haematoma some days after fracture impairs bone healing.[8] In our previous study, we demonstrated that hematomas found at a long bone fracture site contain multi-lineage mesenchymal progenitor cells (long bone fracture haematoma-derived cells; LBHCs), and suggested that the haematoma could be involved in both endochondral and intramembranous ossification.[9,10] Recently, we reported that cells isolated from a mandibular fracture haematoma (mandibular fracture haematoma-derived cells; MHCs) have osteogenic differentiation capacity, and suggested the contribution to intramembranous bone healing. We also demonstrated that the chondrogenic potential of MHCs is inferior to that of long bone bone-marrow stromal cells (BMSCs). These findings indicate that MHCs could be a local reservoir and source of osteogenic progenitors involved in the intramembranous ossification process of mandibular fractures.[11]

Low intensity pulsed ultrasound (LIPUS) is mechanical stimulation that can be transmitted into biological tissues as high-frequency acoustic pressure waves. It has generally been recognized that the micromechanical strains generated by these pressure waves evoke biochemical events that can regulate fracture healing.[12,13] LIPUS stimulation is a clinically established, widely used, and US Food and Drug Administration (FDA) approved intervention for accelerating bone formation during the healing of long bone fractures and non-unions.[13–15] Although there have been many animal studies investigating the effects of LIPUS on mandibular fractures, as well as on distraction osteogenesis, there is currently no clinical adaptation of the use of LIPUS for mandibular fractures.[16–18] LIPUS has been shown to stimulate the osteogenic differentiation of a variety of cells including BMSCs, periosteal cells, and osteoblasts in vitro.[19–21] In our previous study, the osteogenic differentiation capacity was increased when LBHCs were exposed to LIPUS.[22,23] It is known that the fracture healing process differs between long bones (endochondral and intramembranous ossification) and mandibular bones (mainly intramembranous ossification). To date, there have been no studies investigating the effect of LIPUS on MHCs.

We report herein our investigation of the effect of LIPUS on MHCs in order to explore the possibility of applying LIPUS treatment to mandibular fractures, based on our hypothesis that the osteogenic activity of MHCs would be increased by LIPUS.

Methods

Specimens of mandibular fracture hematomas were obtained from five patients with a mean age of 26.4 years (range 15–65 years) during osteosynthesis, at a mean of 4 days (range 1–7 days) after injury. In the delayed case, we spent time scrutinizing the patient's general condition prior to treatment. This delay was not due to the study. The fracture involved the median mandible in three patients and the mandibular body in the other two. Patients taking anticoagulants, steroids, or non-steroidal anti-inflammatory drugs within 3 months before the injury were excluded. This study had ethics committee approval, and informed consent was obtained from all of the patients.

Isolation and culture of MHCs

MHCs were isolated and cultured as described previously.[11] Briefly, the fracture haematoma that had formed fibrin clots was removed manually before any manipulation or irrigation, and was placed in a sterile polypropylene container in order to avoid contamination during the operation. The mean wet weight of the hematomas obtained was 1.03 g (range 0.30–1.94 g). 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 mmol/l L-glutamine (Gibco BRL, Grand Island, NY, USA), and antibiotics. The cultures were incubated in growth medium at 37 °C with 5% humidified carbon dioxide. Approximately 2–3 weeks later, the adherent cells were harvested with 0.05% trypsin containing 0.02% ethylenediaminetetraacetic acid (EDTA; Wako, Osaka, Japan) and were passaged into flasks. Cells that had undergone one to three passages were used in the subsequent assays.

LIPUS treatment

We used a LIPUS exposure device (Teijin Pharma Ltd, Tokyo, Japan) that was adapted for a six-well tissue – cell culture plate in the in vitro experiments.[22–24] This was set at a 1.5 MHz wave with a pulse duration of 200 μs, a repeating pulse at 1 kHz, and an 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). Briefly, 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. The culture plate was then placed on the ultrasound transducer with a thin layer of water to maintain contact. In the LIPUS treatment group (LIPUS (+) group), LIPUS was applied through the bottom of the culture plates for 20 min daily at 37 °C. Cells without LIPUS stimulation served as a control group (LIPUS (−) group).

Cell proliferation

A total of 5×10^4 MHCs per well was seeded into a six-well plate and stationary cultured for 3 days. LIPUS stimulation was applied according to the protocol described, for 4 or 8 days. The growth medium was used for both groups. MHCs were detached using 0.05% trypsin with 0.02% EDTA. The number of MHCs was counted twice using a hemocytometer (Bio-Rad Laboratories Japan, Tokyo, Japan) and the mean number was calculated. The cell viability was found to be >99% by Trypan Blue dye (Gibco BRL) exclusion technique. The results are presented as the relative percentages of the total number of viable cells compared with day 0 (considered to be 100%).

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

Total RNA was extracted from the harvested cells on days 0, 4, 8, 14, and 20 at 1 h after the LIPUS or sham treatment, using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) in accordance with the manufacturer's instructions. Total RNA was reverse-transcribed into single-stranded cDNA using a high-capacity cDNA reverse transcription kit (Applied