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Research Paper
Bone Healing

The osteogenic activity of human mandibular fracture haematoma-derived progenitor cells is affected by bisphosphonate in vitro

Y. Imai, T. Hasegawa, D. Takeda, M. Akashi, T. Komori
Department of Oral and Maxillofacial Surgery, Kobe University Graduate School of Medicine, Kobe, Japan

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Abstract. It is known that bisphosphonates (BPs) suppress the activity of osteoclasts; however, it has not been reported whether BPs affect the potential of human mandibular fracture haematoma-derived cells (MHCs) for bone differentiation. In this study, we examined whether the degree of bone differentiation changes following the administration of BP in vitro. The effects of alendronate and risedronate (10^{-8} to 10^{-7} M (mol/l)) on cell proliferation were evaluated at 4 and 8 days, after which BP treatment was applied for 4, 8, 14, and 20 days prior to assessing the alkaline phosphatase (ALP) activity and performing the mineralization assay. Alendronate 10^{-8} and 10^{-7} M and risedronate 10^{-7} M decreased the degree of cell proliferation on day 8 ($P < 0.05$). Using an ELISA, the ALP activity of the control, alendronate 10^{-8} M, risedronate 10^{-8} M, and risedronate 10^{-7} M groups were 112.1 ± 10.2%, 156.1 ± 24.3%, 138.8 ± 16.5%, and 133.3 ± 10.3%, respectively, at 14 days after treatment (day 0 in each group was considered to be 100%). ALP activity was significantly higher in the alendronate 10^{-8} M and risedronate 10^{-8} and 10^{-7} M groups than in the control group ($P = 0.010$, 0.014, and 0.009, respectively). It is possible that BPs increase the potential of MHCs for osteogenic differentiation depending on the concentration of the drug.

Keywords: mandibular fracture; haematoma; bisphosphonate; alendronate; risedronate; osteogenic activity.

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Bisphosphonates (BPs) constitute a group of pharmacological agents with anti-osteoclastic and antiresorptive properties that are used to treat calcium metabolism disorders, such as osteoporosis, multiple myeloma, Paget's disease, and hypercalcemia of malignancy.[1] BPs exhibit high affinity for bone minerals, preferentially binding to the bone surface at sites of active remodelling.[2]

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The amount of BP taken up by the skeletal system depends on several factors, particularly renal function and the rate of bone turnover.[3] Studies on osteoclasts reacting with BPs have been reported, and the effects of these agents on both osteoclasts and the surrounding cells have been investigated. For example, several studies have found that BPs affect different tissues and cell types, and that bone turnover is suppressed excessively by the administration of these drugs. Furthermore, these agents appear to decrease capillary formation and inhibit various endothelial growth factors.[4,5] BPs inhibit osteogenic cells, osteoclasts, and human fibroblasts, and restrict both vasculogenesis and angiogenesis by inhibiting the cell function of endothelial progenitors and mature endothelial cells.[6–8] In addition, BPs reduce the viability of oral keratinocytes, corresponding to impaired mucosal wound healing, and a reduction in extracellular matrix protein production in patients treated with these drugs has been described.[9,10] In this manner, BPs affect the surrounding tissue and cells. However, despite the variety of reports, no previous study has assessed the effects of BPs on mesenchymal cells of human mandible origin.

It is known that fracture haematomas play an important role in fracture healing, and we have previously reported that mandibular fracture haematoma-derived cells (MHCs) have an effect on the mandible fracture healing process. We have also previously demonstrated that the chondrogenic potential of MHCs is inferior to that of long bone marrow stromal cells. These findings indicate that MHCs may serve as a local reservoir and source of osteogenic progenitors for intramembranous ossification of mandibular fractures.[11,12] Therefore, it may be possible to more accurately assess the effects of BPs on the mandible using human mandible-derived MHCs.

It has been reported that the onset of bisphosphonate-related osteonecrosis of the jaw (BRONJ) in the mandible is more frequent than that observed in the maxilla.[4] In addition, the relationship between BPs and osteoclasts has been examined. However, there are no reports on the effects of BPs on MHCs in the human mandible. We therefore examined the bone differentiation potential and cell proliferative capacity of MHCs treated with alendronate and risedronate.

Materials and methods

Specimens of mandibular fracture haematomas were obtained from five patients during osteosynthesis, at a mean of 2.6 days (range 1–9 days) after injury; their mean age was 46.8 years (range 29–68 years). In the delayed case, we spent time scrutinizing the patient's general condition prior to treatment. The delay was not due to this study. All fractures occurred in the mandibular body. Patients with a history of treatment with anticoagulants, steroids, or non-steroidal anti-inflammatory drugs within 3 months prior to injury were excluded. The ethics committee of the university hospital approved this study and informed consent was obtained from all subjects.

MHC in vitro model

MHCs were isolated and cultured as described previously.[11] Briefly, fracture haematomas formed from fibrin clots were removed manually before manipulation or irrigation and placed in a sterile polypropylene container in order to avoid contamination during the procedure. The mean wet weight of the haematomas obtained was 1.06 g (range 0.10–2.50 g). The specimens were cut into small pieces with a scalpel in growth medium and α-modified minimum essential medium (Sigma, St Louis, MO, USA) containing 10% heat-inactivated foetal bovine serum (Sigma), 2 mmol/l (mM) of L-glutamine (Gibco BRL, Grand Island, NY, USA), and antibiotics. The samples were then 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 passaged into flasks. Cells that had undergone one to three passages were used in the subsequent assays.

MHCs were treated with two BPs diluted in sterile phosphate buffered saline (PBS): alendronate (10^{-8} to 10^{-7} M Fosamax; Merck, Rahway, NY, USA) and risedronate (10^{-8} to 10^{-7} M Actonel; Proctor & Gamble, Cincinnati, OH, USA).[13] Osteogenic medium with dexamethasone (10^{-8} M; Sigma, St. Louis, MO, USA), 10 mM β-glycerophosphate (Sigma), and 50 μg/ml of ascorbic acid was added. The culture medium and drugs were replaced twice a week, and the experiments were terminated at 4, 8, 14, and 20 days after the initiation of the culture.

Cell proliferation

A total of 5×10^4 MHCs per well was seeded into a six-well plate and stationary cultured for 3 days. The effects of alendronate and risedronate (10^{-8} to 10^{-7} M) on cell proliferation were evaluated at 4 and 8 days. Growth medium was used in both groups. The MHCs were subsequently detached using 0.05% trypsin with 0.02% EDTA. The number of MHCs was then counted twice using a haemocytometer (Bio-Rad Laboratories Japan, Tokyo, Japan) and the mean value was calculated. The degree of cell viability was >99% according to the 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%).

Alkaline phosphatase (ALP) activity assay

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 Disruptor XL2000 (Misonix, Farmingdale, NY, USA), and stored at −20 °C until the assay. ALP activity was subsequently assayed according to the release of p-nitrophenol from p-nitrophenylphosphate at a pH of 9.8, and the amount of p-nitrophenol released was monitored based on the optical density at 405 nm using the SensoLyte pNPP Alkaline Phosphatase Assay Kit (AnaSpec Inc., San Jose, CA, USA). The protein concentrations in the sonicated samples were then measured using the BCA Protein Assay Kit (Pierce Biotechnology Inc., Rockford, IL, USA). The results are expressed as the amount of p-nitrophenol produced in nmol/min/mg of protein. The expression levels are expressed relative to the day 0 control culture levels.

Mineralization assay

On day 20, the cells were fixed for 1 h at room temperature in 95% ethanol. The plates were subsequently stained with 1% Alizarin Red S (Hartman-Leddon, Philadelphia, PA, USA) at pH 4.0 for 5 min, washed with water, and dried.

Statistical analysis

Stat View-J 4.5 software programme (Hulinks Inc., Tokyo, Japan) was used for the statistical analysis. The data are presented as the mean and standard error (SEM). Wilcoxon's rank-sum test was used to assess differences in the means between the treated and control cells. A *P*-value of <0.05 was considered to be statistically significant.

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