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ORIGINAL ARTICLE

Effect of bisphosphonate alendronate on ERK-dependent growth in human gingival fibroblasts

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Abstract

Objective: Medication-related osteonecrosis of the jaw (MRONJ) is a complication of bisphosphonate (BP) therapy. The pathophysiology of MRONJ remains unclear. Extensive studies have been performed to examine the effect of BPs on bone metabolism. Breakdown of mucosa is a factor classified into five stages in MRONJ, indicating an important role of oral soft tissue in the pathogenesis of MRONJ.

Methods: We investigated the effect of alendronate, one of the most commonly used BPs worldwide, on the proliferation of human gingival fibroblasts by MTT assay, western blotting, and wound healing assay.

Results: We observed time- and concentration-dependent inhibition of fibroblast proliferation by alendronate. Wound healing assays also showed that alendronate prolonged wound healing in a concentration-dependent manner. MAP kinase signaling pathway exhibits a cardinal role in cell proliferation; however, little is known about whether BPs affect classical MAP kinase ERK in fibroblasts. We found that U0126, a selective inhibitor of MAP kinase kinase (MEK1/2), inhibited fibroblast proliferation similarly to alendronate. Alendronate was also found to inactivate ERK1/2, both of which are downstream molecules of MEK1/2.

Conclusion: our findings indicated that alendronate inhibits the proliferation of human gingival fibroblasts via inactivation of ERK1/2.

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Key words: MRONJ; alendronate; Bisphosphonate; gingival fibroblasts; MAP kinas.

Introduction

Bisphosphonates (BPs) are drugs used to treat bone disorders, including bone metastasis of malignant tumors, osteoporosis, and Paget's disease[1)]. BPs are pyrophosphate (P-O-P) analogs in which the central oxygen is replaced by a carbon (P-C-P) and therefore show resistance to hydrolysis[2)]. Due to their stability and affinity for hydroxyapatite, BPs are easily incorporated into bone[3)]. In addition to their high affinity for bone, BPs have been shown to influence the function of osteoclasts. BPs inhibit osteoclast attachment to the bone surface and induce apoptosis of osteoclasts[4)]. Osteoclasts secrete acids and enzymes that both dissolve bone minerals, leading to bone absorption[5)]. Thus, BPs interfere with bone resorption by osteoclasts.

To date, BPs have been divided into two groups depending on the presence or absence of amine groups. First-generation non-amino-containing BPs (non-N-BPs) (e.g., etidronate, clodronate, and tiludronate) are characterized by alkyl, halide, or hydroxyl side chains[6)]. The second- and third-generation amino-containing BPs (N-BPs) (e.g., alendronate, risedronate,

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ibandronate, pamidronate, and zoledronate) contain an amino group in the side chain to the carbon at the P-C-P structure. Both types of BPs induce osteoclast apoptosis; however, they function differently. Non-N-BPs are incorporated into ATP to form nonhydrolysable analogs of ATP, resulting in the inhibition of ATP-dependent intracellular pathways in osteoclasts[3]. In contrast, N-BPs interfere with the mevalonate biosynthetic pathway to induce osteoclast apoptosis[7]. Recently, N-BPs have been widely used in clinical practice because of their higher bone affinity and greater bone resorption effect[8]. Alendronate is an N-BP that is the most common drug prescribed for osteoporosis.

Following the treatment for osteoporosis with BPs, certain patients developed osteonecrosis of the jaw after experiencing mechanical trauma, for example, tooth extraction[9, 10]. This condition is called bisphosphonate-related osteonecrosis of the jaw (BRONJ). With the increase in the clinical use of BPs, the onset of BRONJ is indeed increasing. Recently, the onset of osteonecrosis of the jaw has been shown to be associated with bone modifying agents (BMAs), including BPs, zoledronic acid, and denosumab. Therefore, the American Association of Oral and Maxillofacial surgeons (AAOMS) recommended changing the nomenclature from BRONJ to medication-related osteonecrosis of the jaw (MRONJ)[11]. After tooth extraction, the socket is filled with a blood clot. Within 1 to 3 weeks, this clot is absorbed by neutrophils or macrophages, and it is replaced by granulation tissue from wound margins of soft tissue or jawbone[12]. Subsequently, granulation tissue is replaced by new bone proliferated from the bone of the socket walls. New bone matures to alveolar bone, and eventually, bone marrow is formed 5 weeks after tooth extraction[13]. MRONJ is a pathological condition characterized by an exposed jawbone in the oral cavity in patients with previous or ongoing BMA[14].

Fibroblasts are differentiated from mesenchymal cells and generate precursors of collagen (procollagen) and elastin (tropoelastin)[15]. The main body of granulation tissue consists of collagen fibers[16]; therefore, fibroblasts play an important role in wound healing of extraction sockets. Previous research has proven that BPs affect not only bone itself but also fibroblasts. BPs inhibit fibroblast growth in vitro[17, 18]. This effect is considered to be a cause of MRONJ onset. However, the mechanism by which BPs inhibit human fibroblast growth has not been elucidated.

Cellular stresses, including UV, ionizing irradiation, oxidative stress, and chemotherapy, influence cell proliferation, apoptosis, inflammation, and immune reactions[19]. During such stress responses, intracellular signaling circuits are activated. Mitogen-activated protein kinase (MAPK) is known as one of the cardinal intracellular signaling circuits[20]. MAPK is mainly classified into three groups: extracellular signal-related kinase (ERK), c-Jun N-terminal kinase (JNK), and p38[21]. Among the MAPK family, the ERK pathway has been shown to be associated with cell proliferation and differentiation[22]. However, little is known about the involvement of ERK in the BP-mediated inhibition of fibroblast proliferation.

In this study, we investigated the effect of alendronate, the most common N-BP in osteoporosis, on the proliferation of human gingival fibroblasts. We found that alendronate inhibits the proliferation of fibroblasts through the inactivation of ERK.

Materials and Methods

1) Cell culture

Human gingival fibroblasts were isolated from healthy gingival tissues of three patients who underwent minor oral surgery at Hirosaki University Hospital. All patients gave written informed consent before providing the samples.

This study was approved by the Committee of Medical Ethics of Hirosaki University School of Medicine, Hirosaki, Japan (approved #2012-058). The isolation of fibroblasts was previously shown[23]. Briefly, gingival tissues were washed in phosphate-buffered saline (PBS, pH 7.4) and cut into small pieces, which were cultured in α-minimal essential medium (MEM, Sigma-Aldrich, St. Louis, MO) containing 10% fetal calf serum (Thermo Fisher Scientific, Waltham, MA), penicillin (100 U/mL), streptomycin (100 μg/mL) and gentamicin (80 μg/mL) for 2 weeks in an atmosphere of 95% air and 5% CO2 at 37° C. When cells growing out from the explants had reached confluence, they were subcultured, and experiments were performed from the third to sixth passages. In experiments with alendronate, the cells were treated with alendronate sodium salt trihydrate (Fujifilm Wako, Tokyo, Japan) dissolved in distilled water. For determination of the effect of the ERK signaling pathway, the cells were treated with the MEK1/2 inhibitor U1026 (Promega, Madison, MI).

2) Cell proliferation assay

Cell viability was measured by using a Cell Counting Kit-8 (Dojindo, Tokyo, Japan) according to the manufacturer's protocol with slight modifications. The assay is based on the conversion of WST-8 [2-(2-methoxy-4-nitrophenyl) -3- (4-nitrophenyl)-5-(2,4-disulfophenyl) -2H-tetrazolium, monosodium salt] to a formazan (a purple dye) by metabolically active cells. The cellular reduction reaction involves the pyridine nucleotide cofactors NADH/NADPH and is only catalyzed by living cells. Gingival fibroblasts were cultured in 96-well culture plates at 2 × 104 cells per well with 100 μL of medium. The cells were treated with a series of concentrations of alendronate up to 72 h. Furthermore, 15 μL/well of the WST-8 reagent was added, and the cells were incubated for 4 h. The absorbance (A570) of the resulting colored solution was measured with a microplate reader (iMark, Bio-Rad, Hercules, CA). Each experiment was performed in triplicate.

3) Wound healing assay

The morphological changes in the cells were observed by using an inverted phase-contrast microscope (Eclipse Ti-E, Nikon, Tokyo, Japan). Live-cell imaging for the wound assay was performed at 37° C by using a top incubator (INUG2-TIZ, Tokai Hit Corporation, Shizuoka, Japan) and a microscope with an X20 objective lens equipped with a spinning disc system (TI-S-EJOY, Nikon) and a CCD camera (CoolSNAP HQ2, Photometrics, Tucson, AZ). A confluent layer of gingival fibroblasts was scratched using a 200 μL pipette tip. Then, time-lapse images were acquired for the indicated times and analyzed by using NIS-Elements AR 4.5 software (Nikon). We measured the scratch area and wound coverage area by ImageJ software (National Institute of Health, USA).

4) Immunoblot analyses

For the immunoblot analyses, we washed the cells twice with PBS and then lysed them in hypotonic lysis buffer [10 mM Tris (pH 7.4), 100 mM NaCl, 1.5 mM $MgCl_2$, and 0.5% NP-40] containing 0.2% protease inhibitors (Sigma-Aldrich). The lysates were cleared by centrifugation at 6,000 rpm for 15 min at 4° C. Aliquots of the supernatants (10 μg) were subjected to electrophoresis on 10% SDS-polyacrylamide gels. The proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA), which were then blocked for 1 h at room temperature in TBST buffer [20 mM Tris (pH 7.4), 150 mM NaCl, 0.1% Tween-20] containing 5% nonfat dry milk (blocking buffer). The membranes were incubated overnight at 4° C with one of the following primary antibodies: mouse anti-total ERK2 (Santa Cruz Biotechnology, Santa Cruz,

CA) or rabbit anti-phospho ERK (Cell Signaling Technology, Beverly, MA). After five washes with TBST, the membranes were further incubated for 1 h at room temperature with HRP-labeled bovine anti-rabbit (Santa Cruz Biotechnology, Santa Cruz, CA) or goat anti-mouse IgG antibodies (Thermo Fisher Scientific) in blocking buffer. The washes were repeated using TBST, and the immunoreactive proteins were then visualized using Luminata Crescendo Western HRP Substrate (Millipore).

5) Densitometric quantification

The integrated optical density of the phosphorylated ERK band was quantified using ImageJ software and was normalized to the total ERK band density.

6) Statistical analysis

The statistical significance of the differences among the group was evaluated by one-way ANOVA. A probability value of less than 0.05 ($P < 0.05$) was considered to indicate statistical significance.

Results

1) Alendronate inhibits the proliferation of gingival fibroblasts

Alendronate has been shown to inhibit the growth of rotator cuff tendon fibroblasts[18]. In gingival fibroblasts, alendronate indeed suppresses cell proliferation[24]. However, the precise effect of alendronate on the proliferation of gingival fibroblasts is poorly understood. Therefore, we initially studied the time- and concentration-dependent effect of alendronate on the proliferation of gingival fibroblasts. Following the incubation of the cells with serial concentrations of alendronate (0-100 μM) for up to 72 h, cell proliferation was evaluated with MTT-based assays (Fig. 1). Ten micromolar alendronate did not affect the proliferation of gingival fibroblasts

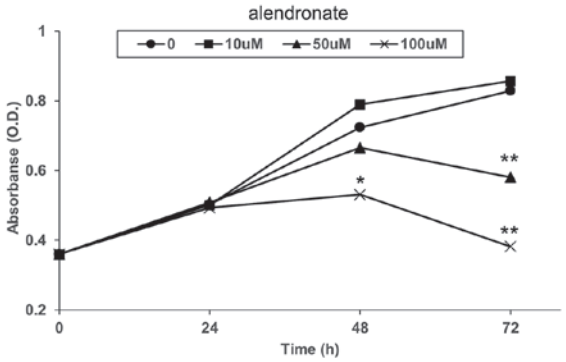

Fig. 1 Effect of alendronate on cell proliferation in gingival fibroblasts. Cells were treated with different alendronate concentrations for up to 72 h. Cell proliferation was quantified by the WST-8 assay. Means ± SDs of three experiments are shown. $^{*}P < 0.05$, $^{**}P < 0.01$.

for up to 72 h. In contrast, 50 μM alendronate slightly inhibited cell proliferation at 48 h after incubation and significantly inhibited cell proliferation at 72 h. Moreover, 100 μM alendronate significantly inhibited cell proliferation from 48 h after incubation.

We further examined the effect of alendronate on fibroblast proliferation by wound healing assays (Fig. 2A). A confluent layer of gingival fibroblasts was scratched using a 200 μL pipette tip, and then, serial concentrations of alendronate (0-500 μM) were added to the cell culture medium. We observed the scratches for 48 h under time-lapse microscopy. In the absence of alendronate, fibroblasts proliferated and began to cover the wound. When the cells were treated with alendronate, proliferation was suppressed in a concentration-dependent manner. In particular, 500 μM alendronate not only suppressed cell proliferation but also showed cytotoxicity to the cells. Quantitative analysis also showed that wound closure is significantly inhibited by alendronate in a concentration of alendronate-dependent manner (Fig. 2B).

2) Involvement of ERK in the proliferation of gingival fibroblasts

ERK plays an essential role in cell

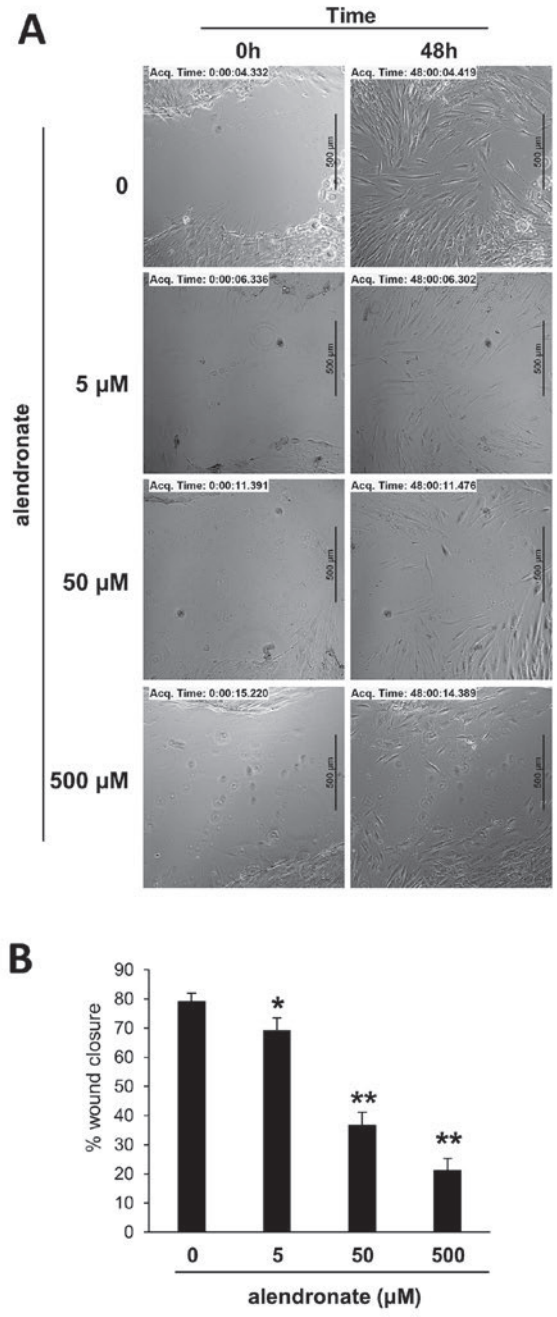

Fig. 2 Wound healing assay of gingival fibroblasts treated with increasing concentrations of alendronate. (A) Confluent monolayers of the cells were scratched as described in the "Materials and Methods". The cells were incubated with a series of concentrations of alendronate for up to 48 h under time-lapse microscopic analysis. The data shown represent three independent experiments. (B) Based on the result in Fig 2A, percent wound closure, exhibited as the area covered by the cells, was quantified. The scratch area at time points 0 h was set to 100 %. Means ± SDs of three experiments are shown
(*P < 0.05, **P<0.01 vs. 0h).

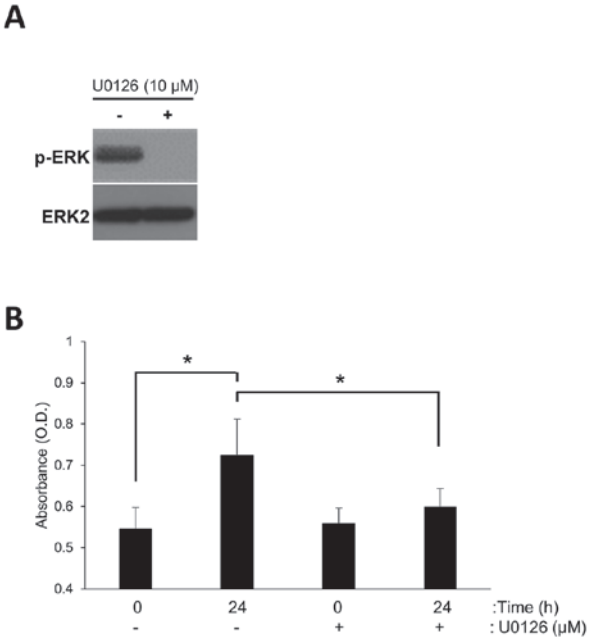

Fig. 3 ERK-dependent cell proliferation of gingival fibroblasts. (A) The cells were treated with U0126 for 1 h. The levels of phosphorylated ERK and total ERK were detected by immunoblot analysis. The results are representative of three independent experiments. (B) The cells were treated with U0126 for 24 h. Cell proliferation was analyzed as described in Fig. 1. *P < 0.05.

proliferation[22]. As expected, we confirmed ERK phosphorylation in human gingival fibroblasts with FBS-containing medium (Fig. 3A). When the cells were pretreated with U0126, a MEK 1/2 inhibitor[25], the phosphorylation of ERK was completely abolished. Similarly, the proliferation of gingival fibroblasts was suppressed by the MEK1/2 inhibitor (Fig. 3B), suggesting that the proliferation of gingival fibroblasts is ERK-dependent.

3) Effect of alendronate on ERK phosphorylation in gingival fibroblasts.

We next examined the effect of alendronate on the activation of ERK in gingival fibroblasts. Treatment of the cells with 100 μM alendronate decreased the levels of ERK phosphorylation in a time-dependent manner (Fig. 4A). Densitometric analysis of immunoblots revealed that the significant suppression of the phosphorylated

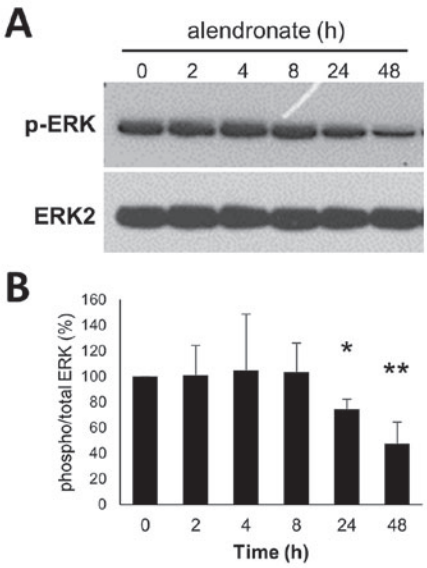

Fig. 4 Time-dependent suppression of ERK phosphorylation by alendronate. Gingival fibroblasts were treated with 100 μM for up to 48 h. (A) The levels of phosphorylated ERK and total ERK were detected by immunoblot analysis. The results are representative of three independent experiments. (B) The integrated optical density of the protein band for phosphorylated ERK in (A) was quantified by using ImageJ and was normalized to that of total ERK (*P < 0.05, **P<0.01 vs. 0h).

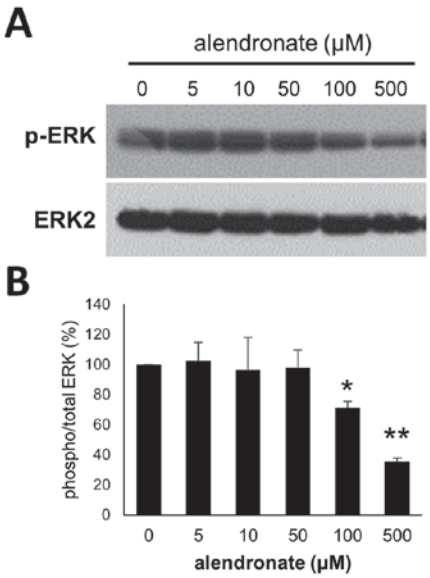

Fig. 5 Concentration-dependent suppression of ERK phosphorylation by alendronate. Gingival fibroblasts were treated with 0-500 μM for 48 h. (A) The levels of phosphorylated ERK and total ERK were detected by immunoblot analysis. The results are representative of three independent experiments. (B) Densitometric quantification of phosphor/total ERK was analyzed as shown in Fig. 4 (*P < 0.05, **P<0.01 vs. control).

form of ERK was observed 8 h after the addition of alendronate (Fig. 4B). A concentration-dependent effect of alendronate on ERK phosphorylation was also observed in gingival fibroblasts (Fig. 5A). Densitometric analysis of immunoblots showed that alendronate treatment significantly lowered ERK2 phosphorylation in a concentration-dependent manner (Fig. 5B).

Discussion

N-BPs, including alendronate, have been shown to affect various types of cells in the oral cavity. For example, short-term observation indicates that N-BPs inhibit bone turnover[26]. Furthermore, long-term exposure to N-BPs suppresses the number of bone progenitor cells[26]. Osteoblasts must adhere to the extracellular matrix (ECM) to exert their cellular function[27]. N-BPs such as pamidronate are known to directly induce osteoblast apoptosis in a dose-dependent manner[28]. In addition, N-BPs inhibit osteoclast proliferation by blocking the adhesion of osteoclasts to hydroxyapatite[29]. Thus, the effects of N-BPs are widely known to modulate bone metabolism. In addition to the effects on osteoclasts, N-BPs inhibit cell migration and induce cell apoptosis in oral epithelial cells and fibroblasts[17]. Indeed, the addition of BPs to oral epithelial cells or oral fibroblasts decreases the total amount of cellular protein production in a concentration of BP-dependent manner[30]. This finding indicates that N-BPs may induce cell apoptosis by affecting global protein translation in those cells.

In the present study, we initially found that alendronate inhibited fibroblast proliferation in a time- and concentration-dependent manner. Sung et al. reported that alendronate also inhibits the proliferation of fibroblasts from human rotator

cull tendons[18]. Although the difference in phenotype between those fibroblasts is unclear, these results suggest that alendronate affects fibroblasts in connective tissue throughout the body. In fact, low-dose systemic application of alendronate reveals healing in the dense connective tissue[31]. These results combined with our results indicate that the dual role of alendronate is concentration-dependent, and that fibroblast proliferation is tightly regulated by the concentration of alendronate.

Fibroblasts are associated with bone metabolism. For instance, synovial fibroblasts play essential roles in the pathogenesis of rheumatoid arthritis (RA) and osteoarthritis (OA) through the production of cytotoxic factors, including inflammatory cytokines and apoptosis inducers[32, 33]. In addition, BPs can induce receptor activator of nuclear factor-κB ligand (RANKL), interleukin-6 (IL-6), and osteoprotegerin (OPG), which can inhibit bone turnover, in human gingival fibroblasts[34]. These results indicate that bisphosphonate may regulate bone metabolism via fibroblasts.

We found that alendronate inhibits the phosphorylation of ERK, a MAPK, in human gingival fibroblasts. MAPKs play essential roles in cell proliferation in response to cytokines, growth factors, and stress[35]. Although alendronate is known to inhibit cell proliferation, little research on how alendronate regulates MAPK has been performed. According to that limited information, alendronate activates ERK in rat bone marrow stromal cells[36]. In these cells, a low concentration (1 μM) of alendronate rapidly (from 60 min) inhibited the phosphorylation of ERK. Our data showed that 10 μM alendronate inhibits cell proliferation of human gingival fibroblasts and inhibits ERK phosphorylation 24 h after the addition of alendronate. These differences may reflect cell type-specific responses to alendronate for cell proliferation. In agreement with our results, alendronate has been shown to induce fibroblast proliferation at concentrations of 10-100 μM[18, 24]. Although the measurement of serum alendronate levels has not been established, alendronate levels in saliva from patients with BRONJ were determined at concentrations of 0.4-5 μM[37]. Thus, we believe that our in vitro analysis would be consistent with the clinical findings.

Alendronate induces phosphorylation of c-Jun N-terminal kinase (JNK) in the MG63 human osteoblast-like cell line[38]. JNK is another group of MAPKs and is known to contribute to apoptotic reactions in response to a variety of stimuli[39]. Taken together with our data, these results indicate that alendronate may mediate the growth inhibition of fibroblasts, at least through the induction of cell death by JNK activation and the inhibition of cell proliferation by ERK inactivation. ERK signaling has been shown to be essential for wound healing in both in vitro and in vivo studies[40]. In addition, a recent study reported that administration of N-BPs prolongs wound closure following tooth extraction in a BRONJ model experiment[41]. Our present study links these studies and suggests that alendronate prolongs the formation of granulation tissue by inhibiting fibroblast proliferation through the inaction of ERK in MRONJ.

Conclusion

Although our present study showed the effect of alendronate on fibroblast proliferation and on inactivation of ERK, the molecular mechanism by which BPs inhibit the activation of ERK is unclear. Future studies will clarify how BPs inhibit the activation of ERK. This data might be useful for clinical application in patients with MRONJ.

Conflicts of interest

All authors have no conflicts of interest

directly relevant to the content of this article.

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