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Alendronate regulates cytokine production induced by lipid A through nuclear factor-κB and Smad3 activation in human gingival fibroblasts

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Background and Objective: Nitrogen-containing bisphosphonates (NBPs) are widely used as anti-bone-resorptive drugs. However, use of NBPs results in inflammatory side-effects, including jaw osteomyelitis. In the present study, we examined the effects of alendronate, a typical NBP, on cytokine production by human peripheral blood mononuclear cells (PBMCs) and gingival fibroblasts incubated with lipid A.

Methods: The PBMCs and gingival fibroblasts were pretreated with or without alendronate for 24 h. Cells were then incubated in the presence or absence of lipid A for a further 24 h. Levels of secreted human interleukin (IL)-1β, IL-6, IL-8 and monocyte chemoattractant protein-1 (MCP-1) in culture supernatants were measured by ELISA. We also examined nuclear factor-κB (NF-κB) activation in both types of cells by ELISA. Activation of Smad3 in the cells was assessed by flow cytometry. In addition, we performed an inhibition assay using SIS3, a specific inhibitor for Smad3.

Results: Pretreatment of PBMCs with alendronate promoted lipid A-induced production of IL-1β and IL-6, but decreased lipid A-induced IL-8 and MCP-1 production. In human gingival fibroblasts, alendronate pretreatment increased lipid A-induced production of IL-6 and IL-8, and increased NF-κB activation in gingival fibroblasts but not PBMCs stimulated with lipid A. In contrast, alendronate activated Smad3 in both types of cells. Finally, SIS3 inhibited alendronate-augmented IL-6 and IL-8 production by human gingival fibroblasts but up-regulated alendronate-decreased IL-8 production by PBMCs.

Conclusion: These results suggest that alendronate-mediated changes in cytokine production by gingival fibroblasts occur via regulation of NF-κB and Smad3 activity.

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Nitrogen-containing bisphosphonates (NBPs) are widely used as anti-bone-resorptive drugs for treatment of osteoporosis, hypercalcemia of malignancy, Paget's disease and tumor-induced bone-related diseases (1–3). The NBPs are taken up by osteoclasts, resulting in osteoclast deactivation and apoptosis. However, NBPs have undesirable side-effects, such as fever, gastrointestinal disturbances, ophthalmic inflammation, jaw osteomyelitis and osteonecrosis (4–8). Intravenous administration of NBPs has also been reported to increase temporarily the plasma levels of proinflammatory cytokines, such as interleukin (IL)-1β and IL-6 (6,7). Proinflammatory cytokines play an important role in the initiation or perpetuation of inflammatory and destructive processes in tissues (9–11). Interleukin-1 and IL-6 also promote differentiation of osteoclast precursors into mature osteoclasts (12,13). In addition, IL-8 and monocyte chemoattractant protein-1 (MCP-1) not only accelerate neutrophil migration but also help to increase osteoclasts (14,15). In contrast, Deng *et al*. (16) demonstrated that IL-1β production was induced in mice injected with lipopolysaccharide (LPS) and alendronate (a typical NBP). Alendronate increased LPS-induced IL-1β production, although this agent alone was not sufficient for IL-1β induction (16). Thus, treatment with NBPs may be a precipitating factor for infectious and inflammatory diseases, including periodontal diseases.

Periodontal diseases are reportedly associated with osteoporosis (17,18). As both of these diseases are prevalent in middle-aged and older people, patients who receive NBP treatment are also likely to have periodontal diseases. Progression of periodontal disease changes the species of bacteria present in the oral cavity. The amount of oral anaerobic gram-negative bacteria, which have LPS in the outer cell wall, increases as periodontal diseases progress (19,20). In fact, endotoxin levels in gingival crevicular fluid are correlated with the severity of clinical and experimental gingival inflammation (21–24). In previous studies, concentrations of endotoxin in gingival washings and dental plaque were expressed in micrograms per millilitre (23,24); however, a study by Fine *et al*. (19) expressed endotoxin levels of the dental pocket in nanograms per millilitre. Lipid A is the bioactive center of LPS and increases the production of proinflammatory cytokines and chemokines by peripheral blood mononuclear cells (PBMCs) and human gingival fibroblasts (25,26). Peripheral blood mononuclear cells play an essential role in some diseases, owing to their ability to secrete proinflammatory cytokines and chemokines in response to stimulation by bacteria and their cell wall components (27,28). Human gingival fibroblasts are the major constituents of periodontal tissue and produce various inflammatory cytokines, such as IL-6 and IL-8, upon stimulation with bacteria and their components (26,29). To elucidate the relationship between oral gram-negative bacteria and NBP-related adverse events, we investigated the effects of alendronate on production of proinflammatory cytokines and chemokines by PBMCs and gingival fibroblasts in response to lipid A at various concentrations.

Nuclear factor-κB (NF-κB) is an important transcription factor implicated in the production of proinflammatory cytokines and chemokines by Toll-like receptor (TLR) ligands, such as LPS and bacterial lipoprotein (30,31). Thus, it is possible that alendronate influences NF-κB activation in human cells. Smad3, another transcription factor, usually helps to inhibit IL-6 expression and IL-8 promoter activity (32,33). However, Smad3 disruption has been reported to accelerate wound healing and improve atopic dermatitis through down-regulation of proinflammatory cytokines, including IL-6 (34,35). Since alendronate can internalize and increase Smad3 levels in mouse cells (36–39), we hereby demonstrate the effects of alendronate on activation of NF-κB and Smad3 in human gingival fibroblasts and PBMCs incubated with lipid A.

Material and methods

Reagents

Alendronate was purchased from LKT Laboratories (St Paul, MN, USA). Synthetic *Escherichia coli* lipid A (compound 506), a typical TLR4 agonist, was purchased from Peptide Institute (Osaka, Japan). Anti-Smad3 antibody and anti-phospho-Smad3 antibody were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). $Pam_3Cys\text{-}Ser\text{-}(Lys)_4$ (Pam_3CSK_4), a TLR2 agonist, was obtained from InvivoGen (San Diego, CA, USA). SIS3, a specific inhibitor of Smad3 (40), was purchased from Calbiochem (Merck KGaA, Darmstadt, Germany) and dissolved in dimethyl sulfoxide (DMSO). All reagents were diluted in medium before use.

Cell culture

Human PBMCs were isolated from heparinized blood of healthy donors by Histopaque-1077 (Sigma, St Louis, MO, USA) density gradient purification. The donors provided written informed consent under a protocol approved by the Institutional Review Board of Ohu University. Cells from the interface were harvested and washed three times in RPMI-1640 medium (Sigma). Collected cells were cultured in RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum (FBS; GIBCO, Carlsbad, CA, USA), 100 units/mL penicillin (GIBCO) and 100 μg/mL streptomycin (GIBCO) in an incubator at 37°C and 5% CO_2.

Human gingival fibroblasts were prepared from clinically inflamed gingival tissue according to a method similar to that described previously (26). Tissue samples were collected from subjects who provided written informed consent under a protocol approved by the Institutional Review Board of Ohu University. Explants were cut into pieces and cultured in six-well flat-bottomed plates (Falcon™; BD, Franklin Lakes, NJ, USA) in α-minimal essential medium (α-MEM; Sigma) supplemented with 10% FBS, L-glutamine (Sigma), penicillin (100 units/mL),

and streptomycin (100 μg/mL). Medium was changed every 3 d for 14–20 d until confluent cell monolayers were formed. After three to four subcultures by trypsinization, homogeneous, slim, spindle-shaped cells growing in characteristic swirls were obtained. Cells were used as confluent monolayers at subculture levels five through eight.

Cytokine measurements

Human PBMCs (1×10^6 cells per well) were pretreated with or without 100 μM alendronate for 24 h. Adherent and nonadherent cells were washed twice with serum-free RPMI-1640 in 24-well flat-bottomed plates (Falcon™). Cells were then incubated in the presence or absence of lipid A (1–100 ng/mL) in RPMI-1640 containing 10% FBS for 24 h. Levels of secreted human IL-1β, IL-6, IL-8 and MCP-1 in culture supernatants were measured by ELISA (IL-1β, IL-6 and MCP-1, eBioscience, San Diego, CA, USA; and IL-8, R&D Systems, Inc., Minneapolis, MN, USA).

Human gingival fibroblasts (2×10^4 cells per well) were pretreated with or without alendronate (1–100 μM) for 24 h and washed twice with serum-free α-MEM in 96-well flat-bottomed plates (Falcon™). Cells were then incubated in the presence or absence of lipid A (100–10,000 ng/mL) in α-MEM containing 10% FBS for 24 h. Levels of secreted human IL-6 and IL-8 in culture supernatants were measured by ELISA. For Smad3 inhibition assays, cells were pretreated with 3 μM SIS3 for 1 h, prior to the addition of alendronate.

Nuclear protein extraction and NF-κB transactivation assay

Cells were incubated in medium with or without 100 μM of alendronate for 24 h, washed twice with medium, and incubated with or without lipid A (1 μg/mL) for 5 h. Nuclear protein extracts of the cells were then prepared with the TransAM™ nuclear extract kit according to the manufacturer's protocol (Active Motif Japan, Tokyo, Japan). Briefly, human gingival fibroblasts were scraped into phosphate-buffered saline (PBS) with phosphatase and protease inhibitors, centrifuged, resuspended in 1 × hypotonic buffer and kept on ice for 15 min. After addition of detergent, lysates were centrifuged at 14,000*g* for 30 s. Pellets were resuspended in complete lysis buffer (20 mM Hepes, pH 7.5, 350 mM NaCl, 20% glycerol, 1% Igepal CA630, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM dithiothrietol, and phosphatase and protease inhibitors) and vortexed. After incubation on ice and centrifugation, supernatants were collected and protein concentration was determined using the BCA™ protein assay kit (Pierce, Rockford, IL, USA). Nuclear factor-κB activation was determined by the TransAM ELISA kit (Active Motif Japan). This assay is based on a colorimetric reaction, and is an alternative to electrophoretic mobility-shift assay. Oligonucleotides containing NF-κB consensus binding sites were immobilized in each well of a 96-well plate. To each well was added 14 μg of nuclear extract, followed by incubation of the plate for 1 h with moderate agitation. Wells were then washed three times with washing buffer (100 mM phosphate buffer, pH 7.5, 500 mM NaCl and 1% Tween 20) and incubated with p50 antibody (1:1000 dilution in washing buffer) for 1 h at room temperature. Wells were finally incubated for 1 h with diluted horseradish peroxidase-conjugated antibody (1:1000 dilution in washing buffer) before addition of 100 μL of developing solution (3,3′,5,5′ tetramethylbenzidine substrate solution diluted in 1% of DMSO) and a 5 min incubation. The reaction was stopped by addition of 0.5 M H_2SO_4 solution. Absorbance was read on a spectrophotometer at 450 nm with a reference wavelength of 655 nm.

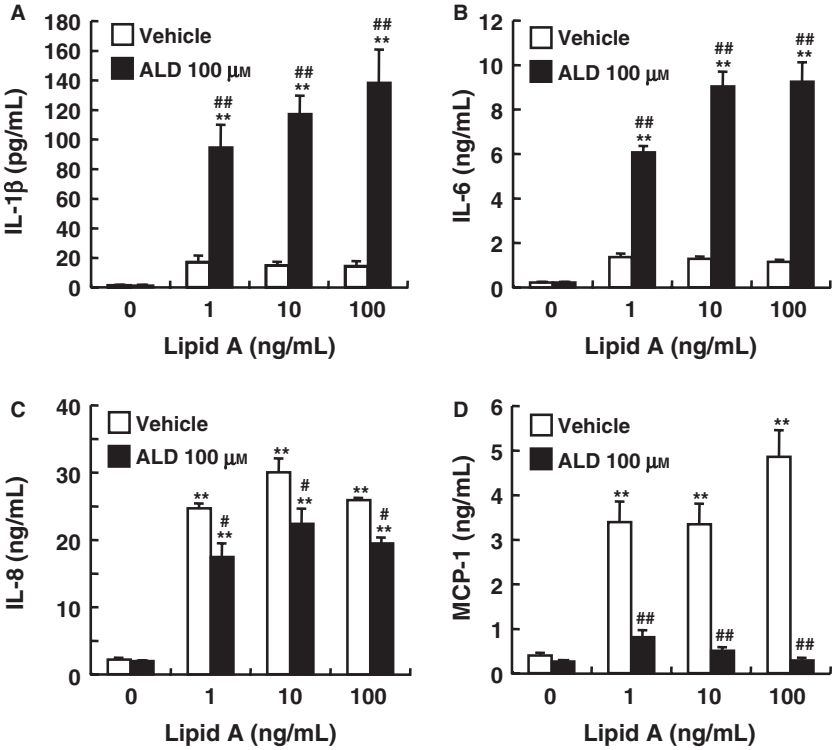

Fig. 1. Effects of pretreatment with alendronate on proinflammatory cytokine and chemokine production by PBMCs incubated with lipid A. The PBMCs were incubated in medium with or without 100 μM alendronate (ALD) for 24 h, washed twice with medium, and incubated with or without the indicated concentrations of lipid A for 24 h. Culture supernatants were collected, and levels of interleukin (IL)-1β (A), IL-6 (B), IL-8 (C) and monocyte chemoattractant protein-1 (MCP-1; D) were measured by ELISA. Results are presented as the means + SEM of triplicate cultures obtained from three independent experiments. $^{**}p < 0.01$ compared with medium alone; $\#p < 0.05$, $\#\#p < 0.01$ compared with lipid A alone.

Flow cytometry

In order to detect Smad3 in human gingival fibroblasts and PBMCs by flow cytometry, cells were treated with or without 100 μM alendronate for 24 h, washed twice with serum-free medium, and incubated with lipid A (100 ng/mL or 1 μg/mL) for 24 h. Collected cells were incubated with BD FACS Lysing Solution for 10 min washed with PBS containing 0.5% bovine serum albumin (BSA), and followed by BD FACS Permeabilizing Solution 2 for 10 min according to the manufacturer's instructions prior to addition of anti-Smad3 or anti-phospho-Smad3 antibodies. After washing, collected cells (5×10^5 per sample) were incubated at room temperature for 60 min with rabbit monoclonal antibodies to human Smad3 or phospho-Smad3 [Correction added after online publication 4 August 2010: rabbit monoclonal antibodies to mouse Smad3 or phospho-Smad3 was changed to: rabbit monoclonal antibodies to human Smad3 or phospho-Smad3]. After washing with PBS containing 0.5% bovine serum albumin (BSA), cells were incubated at room temperature for 30 min with Alexa Fluor® 488-conjugated goat anti-rabbit immunoglobulin G (heavy chain and light chain; Invitrogen, Carlsbad, CA, USA). Cells were washed with PBS containing 0.5% BSA, fixed with 1% paraformaldehyde, and analysed as described above.

Data analysis

Data were analysed using one-way analysis of variance and either the Bonferroni or Dunn method. Results are presented as means ± standard error (SE) of triplicate wells. A value of $p < 0.05$ was considered statistically significant.

Results

Alendronate up-regulates IL-1β and IL-6 production but down-regulates IL-8 and MCP-1 production by PBMCs incubated with lipid A

We examined the effects of alendronate on IL-1β and IL-6 production by PBMCs. Treatment with alendronate alone did not influence IL-1β and IL-6 production (Fig. 1A,B), whereas pretreatment with alendronate significantly up-regulated IL-1β and IL-6 production induced by lipid A. In addition, we investigated the effects of alendronate on chemokine production by PBMCs. Alendronate alone did not change IL-8 and MCP-1 production (Fig. 1C,D). However, pretreatment with 100 μM alendronate down-regu-

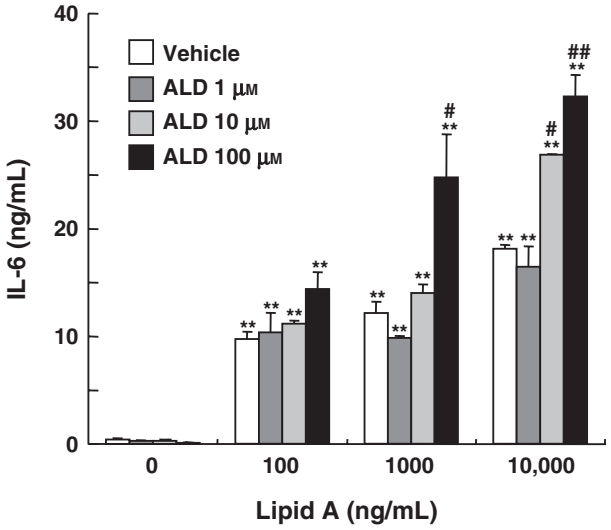

Fig. 2. Effects of alendronate pretreatment on IL-6 production by human gingival fibroblasts incubated with lipid A. Human gingival fibroblasts were incubated in medium with or without the indicated concentrations of alendronate (ALD) for 24 h, washed twice with medium, and incubated with or without the indicated concentrations of lipid A for 24 h. Culture supernatants were collected, and cytokine levels were measured by ELISA. Results are presented as means + SEM of triplicate cultures obtained from three independent experiments. $^{**}p < 0.01$ compared with medium alone; $\#p < 0.05$, $\#\#p < 0.01$ compared with lipid A alone.

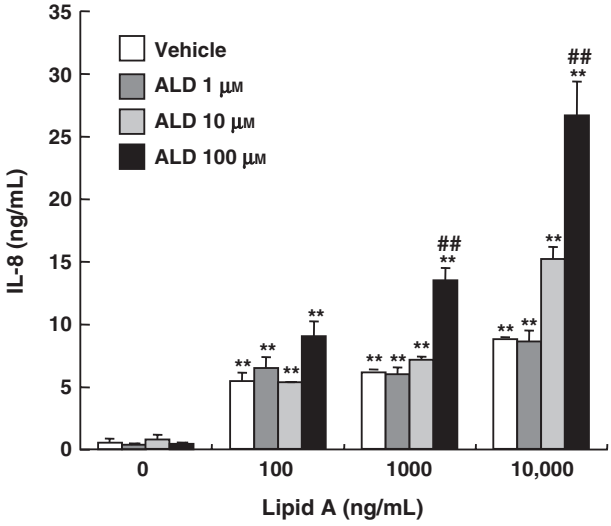

Fig. 3. Effects of alendronate pretreatment on IL-8 production by human gingival fibroblasts incubated with lipid A. Human gingival fibroblasts were incubated in medium with or without the indicated concentrations of alendronate (ALD) for 24 h, washed twice with medium, and incubated with or without the indicated concentrations of lipid A for 24 h. Culture supernatants were collected, and cytokine levels were measured by ELISA. Results are presented as means + SEM of triplicate cultures obtained from three independent experiments. $^{**}p < 0.01$ compared with medium alone; $\#\#p < 0.01$ compared with lipid A alone.

lated IL-8 production induced by lipid A by up to 70%. Furthermore, 100 μM alendronate completely inhibited lipid A-induced MCP-1 production. Similar results were also observed in PBMCs pretreated with alendronate and followed by *Porphyromonas gingivalis* (data not shown).

Alendronate up-regulates IL-6 and IL-8 production by gingival fibroblasts incubated with high concentrations of lipid A

We investigated the effects of alendronate on IL-6 and IL-8 production by gingival fibroblasts. Treatment with alendronate alone did not influence production of these cytokines by the cells in the same manner as PBMCs (Figs 2 and 3). However, pretreatment with 100 μM alendronate up-regulated IL-6 and IL-8 production induced by microgram concentrations of lipid A. This trend was also observed in IL-6 and IL-8 production induced by Pam_3CSK_4, a TLR2 agonist (data not shown).

Alendronate promotes NF-κB activation by gingival fibroblasts incubated with lipid A

We investigated whether alendronate regulated NF-κB activation by gingivalfibroblasts and PBMCs (Fig. 4). Lipid A treatment alone adequately induced NF-κB activation in both types of cells. Pretreatment of gingival fibroblasts but not PBMCs with alendronate, followed by incubation in the presence of 1 μg/mL lipid A, increased NF-κB activation, although alendronate alone did not induce NF-κB activation. Therefore, NF-κB is involved in alendronate-regulated production of IL-6 and IL-8 by human gingival fibroblasts but not by PBMCs.

Smad3 activation is required for alendronate-augmented IL-6 and IL-8 production by human gingival fibroblasts

We next investigated whether alendronate directly activated Smad3. Alendronate enhanced Smad3 activation in gingival fibroblasts incubated with 1 μg/mL lipid A, although total Smad3 levels were not affected (Fig. 5). Alendronate also enhanced Smad3 activation in PBMCs (data not shown). To confirm the role of Smad3 in alendronate-influenced IL-6 and IL-8

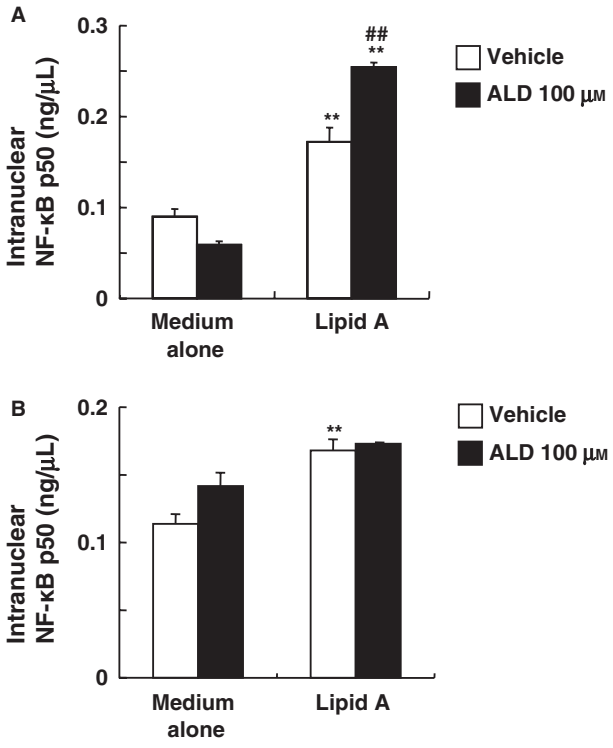

Fig. 4. Alendronate promotes NF-κB activation by lipid A-stimulated human gingival fibroblasts. Human gingival fibroblasts (A) and PBMCs (B) were incubated in medium with or without 100 μM of alendronate (ALD) for 24 h, washed twice with medium, and incubated with or without lipid A (1 μg/mL) for 5 h. The DNA-binding activity of NF-κB p50 in nuclear extracts of gingival fibroblasts was determined using an ELISA-based transcription factor assay. Results are presented as the means ± SEM of triplicate cultures obtained from two independent experiments. $^{**}p < 0.01$ compared with medium alone; $\#\#p < 0.01$ compared with lipid A alone.

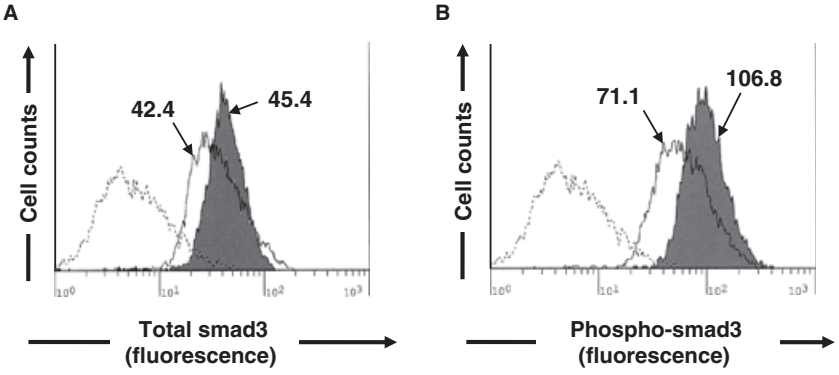

Fig. 5. Smad3 is activated by alendronate in human gingival fibroblasts. Cells were incubated with medium (continuous line) or 100 μM alendronate (shaded area) for 24 h, washed, and incubated for 24 h with lipid A (1 μg/mL). Treated cells were stained with specific antibodies to total Smad3 or phospho-Smad3. Total Smad3 (A) and phospho-Smad3 (B) were analysed by flow cytometry. Samples stained with the secondary antibody alone were used as negative controls (dotted lines). Values indicated in the histograms represent the mean fluorescence intensity of cells based on two independent experiments.

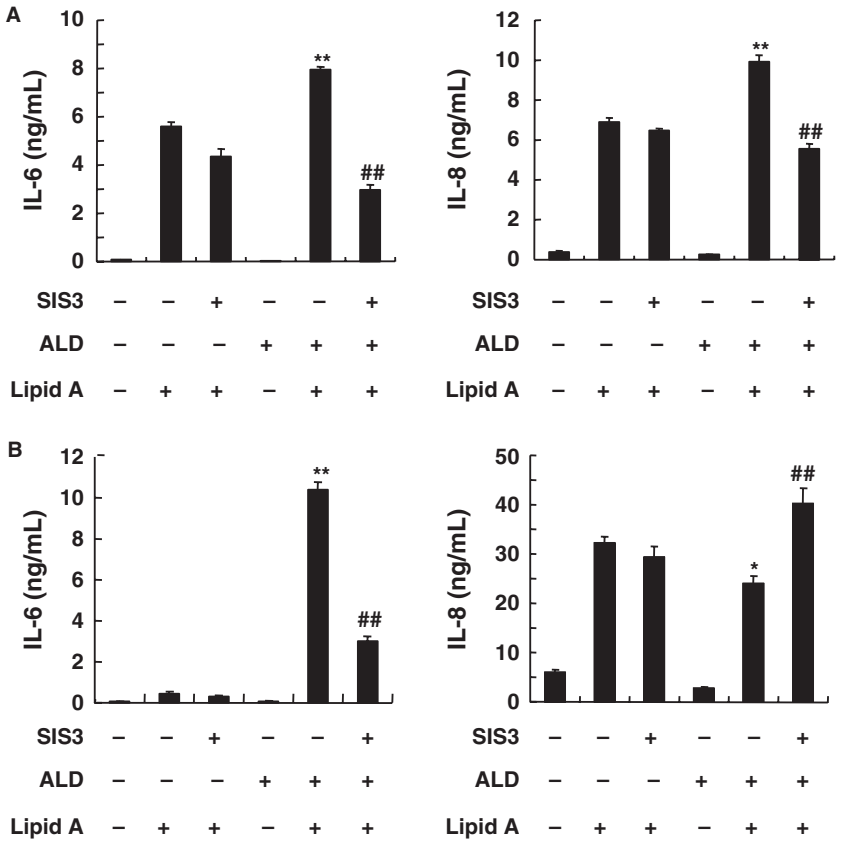

Fig. 6. SIS3 inhibits alendronate-augmented IL-6 and IL-8 production by human gingival fibroblasts. Human gingival fibroblasts (A) and PBMCs (B) were pretreated with 3 μM SIS3 or the same amount of DMSO for 1 h, incubated with medium with or without 100 μM alendronate (ALD) for 24 h, washed twice with medium, and incubated with or without lipid A (A, 1 μg/mL; and B, 100 ng/mL) for another 24 h. Culture supernatants were collected, and levels of IL-6 (left panels) and IL-8 (right panels) were measured by ELISA. Results are presented as the means + SEM of triplicate cultures obtained from three independent experiments. $*p < 0.05$, $**p < 0.01$ compared with lipid A alone, $\#\#p < 0.01$ compared with the ALD and lipid A without SIS3 treatment.

production, we performed an inhibition assay using SIS3, which is a specific inhibitor for Smad3. SIS3 significantly inhibited alendronate-regulated IL-6 and IL-8 production by human gingival fibroblasts (Fig. 6A). These results suggest that alendronate augmented IL-6 and IL-8 production by gingival fibroblasts incubated with high concentrations of lipid A through NF-κB and Smad3 activation. However, SIS3 up-regulated alendronate-decreased IL-8 production in PBMCs (Fig. 6B). These results suggest that Smad3 inhibits IL-8 production in PBMCs.

Discussion

In this study, we found that alendronate pretreatment of PBMCs augmented IL-1β and IL-6 production induced by lipid A. Similar results were obtained in mouse macrophage-like J774.1 cells (41). It was also reported that alendronate plus IL-1β synergistically increased IL-6 production (42). Thus, alendronate may promote efficient production of IL-6 by lipid A. In addition to bacterial components, bone matrix constituents also stimulate IL-1 release from human PBMCs (43). As such, long-term use of alendronate might augment IL-6 production by PBMCs and gingival fibroblasts in the vicinity of bones as well as facilitate osteoclast formation, because IL-6 is required for the differentiation of osteoclast precursors into mature osteoclasts. In fact, long-term alendronate treatment has been reported to increase the number of osteoclasts and cause atypical femoral fracture (44,45). Interleukin-6 also protects neutrophils from apoptosis and sustains the release of proteolytic enzymes in osteomyelitis (46). Thus, alendronate-increased IL-6 production by PBMCs and gingival fibroblasts may contribute to the delay of neutrophil apoptosis and the promotion of tissue damage in the jaw.

Our data also demonstrated that alendronate treatment of PBMCs resulted in decreased IL-8 and MCP-1 production by lipid A. Li *et al.* (47) suggested that osteoclasts and precursor monocytes are recruited to the remodeling site by MCP-1 to initiate the process of bone remodeling. Therefore, alendronate might inhibit normal activation and migration of osteoclasts by down-regulating chemokine production by PBMCs. However, Gazzaniga *et al.* (48) also demonstrated that pharmacological inhibition of MCP-1 with bindarit (2-methyl-2-((1-(phenylmethyl)-1H-indazol-3yl) methoxy) propanoic acid) conferred necrotic tumor masses. Thus, the inhibitory effect of alendronate on MCP-1 production could cause osteonecrosis of the jaw.

The present study demonstrated that alendronate increased IL-8 production by gingival fibroblasts incubated with high concentrations of lipid A. Since NF-κB is an important transcription factor implicated in IL-1, IL-6, IL-8 and MCP-1 production by lipid A (30), it is possible that alendronate regulates NF-κB activation in host cells. Our data demonstrate that alendronate did not change NF-κB activation in PBMCs. Thus, NF-κB does not regulate alendronate-mediated changes in cytokine production by PBMCs. However, as NF-κB activation in response to a combination of alendronate and lipid A treatment was greater in human gingival fibroblasts compared with lipid A treatment alone, we conclude that alendronate can increase IL-6 and IL-8 production by gingival fibroblasts incubated with high concentrations of lipid A *in vitro*. Alendronate treatment may increase production of these cytokines with lower concentrations of lipid A because sera of patients with periodontitis

contain significantly higher levels of soluble CD14, which assists the transfer of lipid A to TLR4, than those of healthy subjects (49–51).

Our data demonstrated that alendronate enhances Smad3 activation. Previous studies have demonstrated that Smad3 usually inhibits IL-6, IL-8 and MCP-1 production (32,33,52). We previously reported that alendronate down-regulated MCP-1 and MIP-1α production induced by synthetic bacterial components via Smad3 activation in J774.1 cells (39). SIS3 up-regulated alendronate-decreased MCP-1 and MIP-1α production by the cells incubated with lipid A. In the present study, we demonstrated that SIS3 up-regulated alendronate-decreased IL-8 production by PBMCs stimulated with lipid A. These results suggest that Smad3 plays a role in the inhibition of chemokine production by J774.1 cells and PBMCs. Our data also demonstrated that pretreatment with SIS3 inhibited alendronate-augmented IL-6 and IL-8 production by gingival fibroblasts and alendronate-augmented IL-6 production by PBMCs. Moreover, Smad3 disruption has been reported to improve atopic dermatitis by down-regulation of IL-6 and reduce incidence of tumor metastasis by inhibition of IL-8 production (35,53). Therefore, the role of Smad3 in IL-6 and IL-8 production differs by the type of cell. Smad3 activation in human gingival fibroblasts has been reported to increase matrix metalloproteinase-13, which is important in degradation of extracellular matrix (54). As such, matrix metalloproteinase-13 production might be augmented by alendronate and thereby accelerate the breakdown of tissues.

Menezes *et al*. (55) reported that alendronate inhibited alveolar bone loss in experimental periodontitis and the growth of periodontal pathogenic bacteria *in vitro*. However, NBPs are deposited in bone, and the half-life of NBPs in bone is very long (56). Thus, our results suggest that alendronate-mediated changes in cytokine production by cells occur via regulation of transcriptional activity, and that long-term use of alendronate may exacerbate infectious diseases, including jaw osteomyelitis, through changes in cytokine production induced by bacterial components.

Acknowledgements

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References

1. Fleisch H. Bisphosphonates, pharmacology and the use in the treatment of tumour induced hypercalcaemia and metastatic bone diseases. *Drugs* 1991;**42:** 919–944.
2. Rogers MJ, Gordon S, Benford HL *et al*. Cellular and molecular mechanisms of action of bisphosphonates. *Cancer* 2000;**88:** 2961–2978.
3. Lipton A. Toward new horizons: the future of bisphosphonates therapy. *Oncologist* 2004;**9:**38–47.
4. Adami S, Bhalla AK, Dorizzi R *et al*. The acute phase response after bisphosphonate administration. *Calcif Tissue Int* 1987;**41:**326–331.
5. Siris E. Bisphosphonates and iritis. *Lancet* 1993;**341:**436–437.
6. Sauty A, Pecherstorfer M, Zimmer-Roth I *et al*. Interleukin-6 and tumor necrosis factor α levels after bisphosphonates treatment *in vitro* and in patients with malignancy. *Bone* 1996;**18:**133–139.
7. Thiébaud D, Sauty A, Burckhardt P *et al*. An *in vitro* and *in vivo* study of cytokines in the acute-phase response associated with bisphosphonates. *Calcif Tissue Int* 1997;**61:**386–392.
8. Marx RE, Cillo JE Jr, Ulloa JJ. Oral bisphosphonate-induced osteonecrosis: risk factors, prediction of risk using serum CTX testing, prevention, and treatment. *J Oral Maxillofac Surg* 2007;**65:** 2397–2410.
9. Lee DM, Weinblatt ME. Rheumatoid arthritis. *Lancet* 2001;**358:**903–911.
10. Charo IF, Taubman MB. Chemokines in the pathogenesis of vascular disease. *Circ Res* 2004;**95:**858–866.
11. Gauldie J, Bonniaud P, Sime P, Ask K, Kolb M. TGF-β, Smad3 and the process of progressive fibrosis. *Biochem Soc Trans* 2007;**35:**661–664.
12. Tamura T, Udagawa N, Takahashi N *et al*. Soluble interleukin-6 receptor triggers osteoclast formation by interleukin 6. *Proc Natl Acad Sci USA* 1993;**90:** 11924–11928.
13. Suda T, Takahashi N, Udagawa N, Jimi E, Gillespie MT, Martin TJ. Modulation of osteoclast differentiation and function by the new members of the tumor necrosis factor receptor and ligand families. *Endocr Rev* 1999;**20:**345–357.
14. Lu Y, Cai Z, Xiao G *et al*. Monocyte chemotactic protein-1 mediates prostate cancer-induced bone resorption. *Cancer Res* 2007;**67:**3646–3653.
15. Hsu YL, Hung JY, Ko YC, Hung CH, Huang MS, Kuo PL. Phospholipase D signaling pathway is involved in lung cancer-derived IL-8 increased osteoclastogenesis. *Carcinogenesis* 2010;**31:** 587–596.
16. Deng X, Yu ZQ, Funayama H *et al*. Mutual augmentation of the induction of the histamine-forming enzyme, histidine decarboxylase, between alendronate and immuno-stimulants (IL-1, TNF, and LPS), and its prevention by clodronate. *Toxicol Appl Pharmacol* 2006;**213:**64–73.
17. Geurs N. Osteoporosis and periodontal disease. *Periodontol 2000* 2007;**44:**29–43.
18. Edwards BJ, Migliorati CA. Osteoporosis and its implications for dental patients. *J Am Dent Assoc* 2008;**139:**545–552.
19. Fine DH, Mendieta C, Barnett ML, Furgang D, Naini A, Vincent JW. Endotoxin levels in periodontally healthy and diseased sites: correlation with levels of gram-negative bacteria. *J Periodontol* 1992;**63:** 897–901.
20. Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL Jr. Microbial complexes in subgingival plaque. *J Clin Periodontol* 1998;**25:**134–144.
21. Simon BI, Goldman HM, Ruben MP, Baker E. The role of endotoxins in periodontal disease. II. Correlation of the quantity of endotoxin in human gingival exudate with the clinical degree of inflammation. *J Periodontol* 1970;**41:** 81–86.
22. Simon BI, Goldman HM, Ruben MP, Baker E. The role of endotoxins in periodontal disease. III. Correlation of the quantity of endotoxin in human gingival exudate with the histologic degree of inflammation. *J Periodontol* 1971;**42:** 210–216.
23. Shapiro L, Lodato FM Jr, Courant PR, Stallard RE. Endotoxin determinations in gingival inflammation. *J Periodontol* 1972;**43:**591–596.
24. Tzamouranis A, Matthys J, Ishikawa I, Cimasoni G. Increase of endotoxin concentration in gingival washings during experimental gingivitis in man. *J Periodontol* 1979;**50:**175–177.
25. Kotani S, Takada H, Tsujimoto M *et al*. Synthetic lipid A with endotoxic and related biological activities comparable to those of a natural lipid A from an *Escherichia coli* re-mutant. *Infect Immun* 1985;**49:**225–237.
26. Tamai R, Sakuta T, Matsushita K *et al*. Human gingival $CD14^+$ fibroblasts primed with gamma interferon increase

production of interleukin-8 in response to lipopolysaccharide through up-regulation of membrane CD14 and MyD88 mRNA expression. *Infect Immun* 2002;**70:** 1272–1278.
27. Fujihashi K, Yamamoto M, Hiroi T, Bamberg TV, McGhee JR, Kiyono H. Selected Th1 and Th2 cytokine mRNA expression by $CD4^+$ T cells isolated from inflamed human gingival tissues. *Clin Exp Immunol* 1996;**103:**422–428.
28. Frostegård J, Ulfgren AK, Nyberg P *et al*. Cytokine expression in advanced human atherosclerotic plaques: dominance of pro-inflammatory (Th1) and macrophage-stimulating cytokines. *Atherosclerosis* 1999;**145:**33–43.
29. Takada H, Iki K, Sakuta T *et al*. Lipopolysaccharides of oral black pigmented bacteria and periodontal diseases. A novel immunomodulator different from endotoxin was extracted from *Prevotella intermedia* ATCC 25611 with hot phenol-water. *Prog Clin Biol Res* 1995;**392:**59–68.
30. Pahl HL. Activators and target genes of Rel/NF-κB transcription factors. *Oncogene* 1999;**18:**6853–6866.
31. Aderem A, Ulevitch RJ. Toll-like receptors in the induction of the innate immune response. *Nature* 2000;**406:**782–787.
32. Kelley TJ, Elmer HL, Corey DA. Reduced Smad3 protein expression and altered transforming growth factor-β1-mediated signaling in cystic fibrosis epithelial cells. *Am J Respir Cell Mol Biol* 2001;**25:** 732–738.
33. Feinberg MW, Watanabe M, Lebedeva MA *et al*. Transforming growth factor-β1 inhibition of vascular smooth muscle cell activation is mediated via Smad3. *J Biol Chem* 2004;**279:**16388–16393.
34. Ashcroft GS, Yang X, Glick AB *et al*. Mice lacking Smad3 show accelerated wound healing and an impaired local inflammatory response. *Nat Cell Biol* 1999;**1:**260–266.
35. Anthoni M, Wang G, Deng C, Wolff HJ, Lauerma AI, Alenius HT. Smad3 signal transducer regulates skin inflammation and specific IgE response in murine model of atopic dermatitis. *J Invest Dermatol* 2007;**127:**1923–1929.
36. Lin JH, Chen IW, Deluna FA. On the absorption of alendronate in rats. *J Pharm Sci* 1994;**83:**1741–1746.
37. Porras AG, Holland SD, Gertz BJ. Pharmacokinetics of alendronate. *Clin Pharmacokinet* 1999;**36:**315–328.
38. Kaji H, Naito J, Inoue Y, Sowa H, Sugimoto T, Chihara K. Statin suppresses apoptosis in osteoblastic cells: role of transforming growth factor-β-Smad3 pathway. *Horm Metab Res* 2008;**40:** 746–751.
39. Masuda T, Deng X, Tamai R. Mouse macrophages primed with alendronate down-regulate monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-1α (MIP-1α) production in response to Toll-like receptor (TLR) 2 and TLR4 agonist via Smad3 activation. *Int Immunopharmacol* 2009;**9:**1115–1121.
40. Jinnin M, Ihn H, Tamaki K. Characterization of SIS3, a novel specific inhibitor of Smad3, and its effect on transforming growth factor-β1-induced extracellular matrix expression. *Mol Pharmacol* 2006;**69:**597–607.
41. Deng X, Tamai R, Endo Y, Kiyoura Y. Alendronate augments interleukin-1β release from macrophages infected with periodontal pathogenic bacteria through activation of caspase-1. *Toxicol Appl Pharmacol* 2009;**235:**97–104.
42. Sanders JL, Tarjan G, Foster SA, Stern PH. Alendronate/interleukin-1β cotreatment increases interleukin-6 in bone and UMR-106 cells: dose dependence and relationship to the antiresorptive effect of alendronate. *J Bone Miner Res* 1998;**13:**786–792.
43. Pacifici R, Carano A, Santoro SA *et al*. Bone matrix constituents stimulate interleukin-1 release from human blood mononuclear cells. *J Clin Invest* 1991;**87:**221–288.
44. Lenart BA, Lorich DG, Lane JM. Atypical fractures of the femoral diaphysis in postmenopausal women taking alendronate. *N Engl J Med* 2008;**358:**1304–1306.
45. Weinstein RS, Roberson PK, Manolagas SC. Giant osteoclast formation and long-term oral bisphosphonate therapy. *N Engl J Med* 2009;**360:**53–62.
46. Asensi V, Valle E, Meana A *et al*. In vivo interleukin-6 protects neutrophils from apoptosis in osteomyelitis. *Infect Immun* 2004;**72:**3823–3828.
47. Li X, Qin L, Bergenstock M, Bevelock LM, Novack DV, Partridge NC. Parathyroid hormone stimulates osteoblastic expression of MCP-1 to recruit and increase the fusion of pre/osteoclasts. *J Biol Chem* 2007;**282:**33098–33106.
48. Gazzaniga S, Bravo AI, Guglielmotti A *et al*. Targeting tumor-associated macrophages and inhibition of MCP-1 reduce angiogenesis and tumor growth in a human melanoma xenograft. *J Invest Dermatol* 2007;**127:**2031–2041.
49. Loppnow H, Stelter F, Schönbeck U *et al*. Endotoxin activates human vascular smooth muscle cells despite lack of expression of CD14 mRNA or endogenous membrane CD14. *Infect Immun* 1995;**63:**1020–1026.
50. Hayashi J, Masaka T, Ishikawa I. Increased levels of soluble CD14 in sera of periodontitis patients. *Infect Immun* 1999;**67:**417–420.
51. Akashi S, Saitoh S, Wakabayashi Y *et al*. Lipopolysaccharide interaction with cell surface Toll-like receptor 4-MD-2: higher affinity than that with MD-2 or CD14. *J Exp Med* 2003;**198:**1035–1042.
52. Feinberg MW, Shimizu K, Lebedeva M *et al*. Essential role for Smad3 in regulating MCP-1 expression and vascular inflammation. *Circ Res* 2004;**94:**601–608.
53. Lu S, Lee J, Revelo M, Wang X, Lu S, Dong Z. Smad3 is overexpressed in advanced human prostate cancer and necessary for progressive growth of prostate cancer cells in nude mice. *Clin Cancer Res* 2007;**13:**5692–5702.
54. Leivonen SK, Chantry A, Hakkinen L, Han J, Kahari VM. Smad3 mediates transforming growth factor-β-induced collagenase-3 (matrix metalloproteinase-13) expression in human gingival fibroblasts. Evidence for cross-talk between Smad3 and p38 signaling pathways. *J Biol Chem* 2002;**277:**46338–46346.
55. Menezes AM, Rocha FA, Chaves HV, Carvalho CB, Ribeiro RA, Brito GA. Effect of sodium alendronate on alveolar bone resorption in experimental periodontitis in rats. *J Periodontol* 2005;**76:** 1901–1909.
56. Fleisch HA. Bisphosphonates: preclinical aspects and use in osteoporosis. *Ann Med* 1997;**29:**55–62.