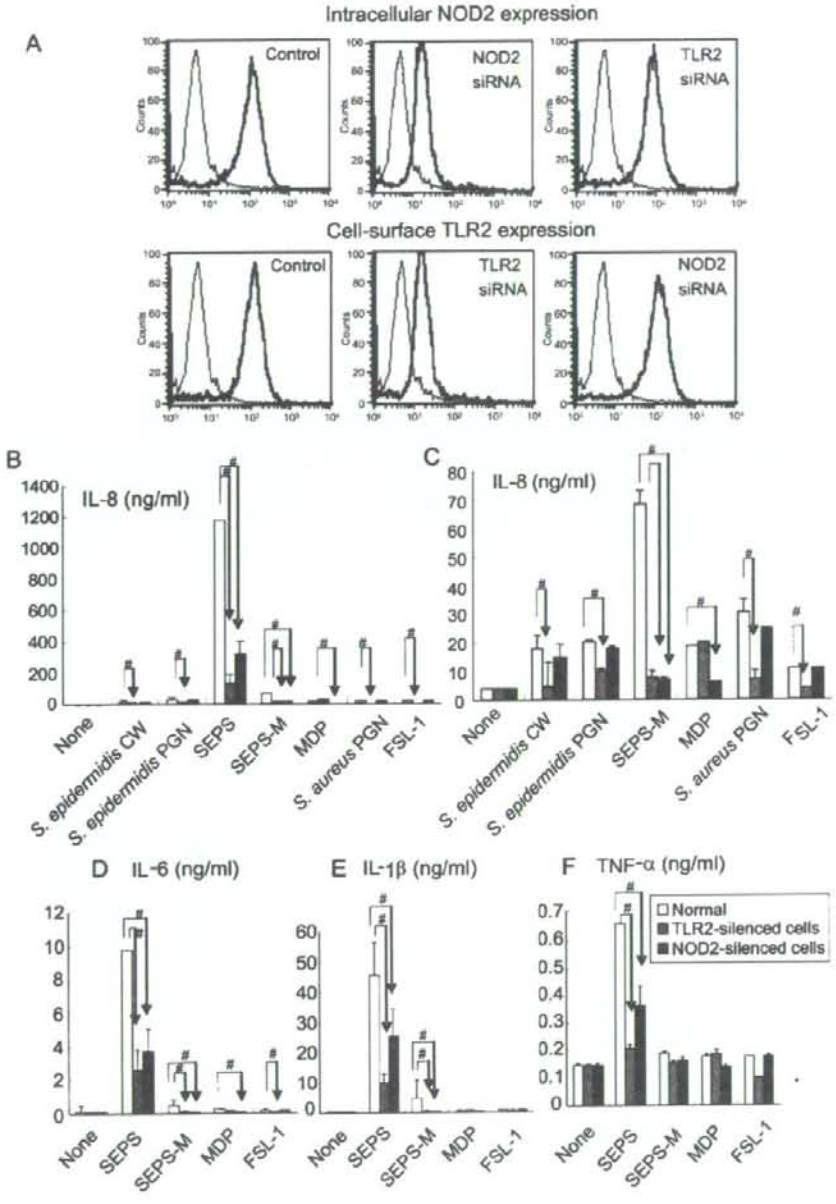

Fig. 5. The marked activities of SEPS were reduced by RNAi assays targeting TLR2 and NOD2. (A) Human monocytic THP-1 cells were transfected with specific siRNA targeting the TLR2 or NOD2 gene. After 24 h, the cells were stained with antibodies specific for NOD2, TLR2, or control IgG at 4°C for 30 min, followed by FITC-conjugated secondary antibodies, and then subjected to flow cytometry. The transfectants and parent cells were stimulated with cell walls, PGNs, SEPS and SEPS-M prepared from *S. epidermidis*, MDP, *S. aureus* PGNs (100 μg/ml for each specimen), and FSL-1 (1 nM). The concentrations of IL-8 (B and C: data other than SEPS are shown in an expanded scale in B), IL-6 (D), IL-1β (E), and TNF-α (F) in the culture supernatants were determined by ELISA. In IL-6, IL-1β and TNF-α assays, SEPS, SEPS-M, MDP and FSL-1 were selected for examination. All assays were carried out in triplicate, and the results are expressed as mean ± SD. The results are representative of three different experiments. $^{\#}P < 0.05$ versus the respective control.

Water-soluble PGN SEPS activated THP-1 cells through TLR2 and NOD2

To elucidate the reason for the outstanding activity of SEPS, we examined the signaling system of SEPS as compared with those of parent PGNs, SEPS-M and related reference specimens by RNAi assays targeting possible receptors for TLR2 and NOD2. The protein levels of NOD2 and TLR2 determined by flow cytometry were significantly suppressed using specific siRNA in THP-1 cells (Fig. 5A). Regarding IL-8 induction, the strong activity of SEPS was markedly reduced both in TLR2- and NOD2-silenced cells (Fig. 5B). It must be noted that the activities of reference synthetic compounds were specifically suppressed in respective transfectants; the activities of NOD2-agonistic MDP and TLR2/6-agonistic FSL-1 were reduced close to the control level in NOD2-silenced and TLR2-silenced cells, respectively (Fig. 5C). In the same experiment, weak but significant activities of the cell walls and PGNs from *S. epidermidis* and the reference *S. aureus* PGNs were significantly suppressed in TLR2-, but not NOD2-, silenced cells. On the other hand, the moderate activity of SEPS-M was significantly reduced in TLR2- as well as NOD2-silenced cells. Next, we examined IL-1β-, IL-6- and TNF-α-inducing activities of SEPS, SEPS-M, MDP, and FSL-1 in the above silenced and parent cells. SEPS exhibited outstanding activities to induce these cytokines, and the strong activities of SEPS were markedly reduced both in TLR2- and NOD2-silenced cells (Fig. 5D–F). SEPS-M exhibited moderate activities to induce IL-6 and IL-1β, and these activities were also significantly reduced both in TLR2- and NOD2-silenced cells similar to the case of IL-8 secretion. Concerning IL-6 induction, weak but significant activities of reference synthetic compounds were significantly suppressed in the respective transfectants similar to the experiments of IL-8-induction; MDP and FSL-1 were significantly suppressed in NOD2- and TLR2-silenced cells, respectively (Fig. 5D). Concerning IL-1β induction, clear reductions were not observed (Fig. 5E), probably because the cytokine levels induced by these compounds were not enough for RNAi assays. Furthermore, TNF-α induction-tested specimens, except SEPS, did not exhibit clear activities to analyze by RNAi assay. These findings clearly demonstrated that water-soluble SEPS activated human monocytic THP-1 cells both in a TLR2- and NOD2-dependent manner, which might induce synergistic effects and resulted in marked activities. On the other hand, water-insoluble cell walls and PGNs activated cells mainly via TLR2, but not NOD2. In the case of SEPS-M, the TLR2-agonistic structure might remain even after M-1 digestion, although the activity was far less than SEPS.

Synergistic activation of THP-1 cells by PGNs in combination with MDP to induce pro-inflammatory cytokines

To examine the possible synergistic effect between TLR2- and NOD2-agonistic moieties in PGNs, OCT-treated THP-1 cells were stimulated with TLR2 agonistic PGNs of *S. epidermidis* or *S. aureus* in combination with NOD2-agonistic MDP, and IL-8 and IL-1β in the culture supernatants were measured after 24 h cultivation. Both PGN preparations in combination with MDP clearly induced the synergistic activation of THP-1 cells to induce IL-8 and IL-1β (Fig. 6).

Discussion

As above mentioned, Uehara *et al.*[20] demonstrated the synergistic activation of human monocytic THP-1 cells to induce IL-8 by combinatory stimulation with various TLR agonists (TLR2, TLR4 and TLR9 agonists) and NOD1 or NOD2 agonist. Van Heel *et al.*[28] also reported that multiple TLR ligands (TLR1/2, 2/6, 4, 5, 7/8 ligands) in combination with a NOD1 agonist induced the synergistic activation of human peripheral blood mononuclear cells to induce the release of various cytokines. In our original study of this lineage, Yang *et al.*[17] first demonstrated the synergistic activation of THP-1 cells by combinatory stimulation with LPS and MDP. Then, Fritz *et al.*[29] reported the synergistic stimulation of human monocytes by TLR4 in combination with either NOD1 or NOD2 agonist; furthermore, they reported a synergism effect on human dendritic cells. Recently, Abbott *et al.*[30] presented a model for the signaling cross-talk between extracellular TLRs and intracellular NOD2. In this model, both TLR and NOD2 signaling induce ubiquitination of NEMO (NF-κB essential modifier), resulting in synergistic activation of NF-κB. In contrast, Watanabe *et al.*[31] reported that NOD2 stimulation specifically down-regulated murine dendritic cells regarding TLR2-mediated T-helper type 1 (Th1) responses in relation to the possible pathogenesis of Crohn disease, in which hyper Th1 responses associated with NOD2 dysfunction have been observed. Tada *et al.*[32] demonstrated synergistic Th1-promoting IL-12 production by human dendritic cells upon combinatory stimulation of NOD1 or NOD2 agonist together with TLR3, TLR4, or TLR9 agonist, whereas no such synergism was observed between TLR2 agonist and NOD1 or NOD2 agonist. Taken together, these findings indicated that various TLR agonists, in combination with NOD1 or NOD2 agonist, induced the synergistic activation of monocytic cells to release pro-inflammatory cytokines, whereas this is not the case with the Th1-promoting responses of dendritic cells, such as IL-12 production,

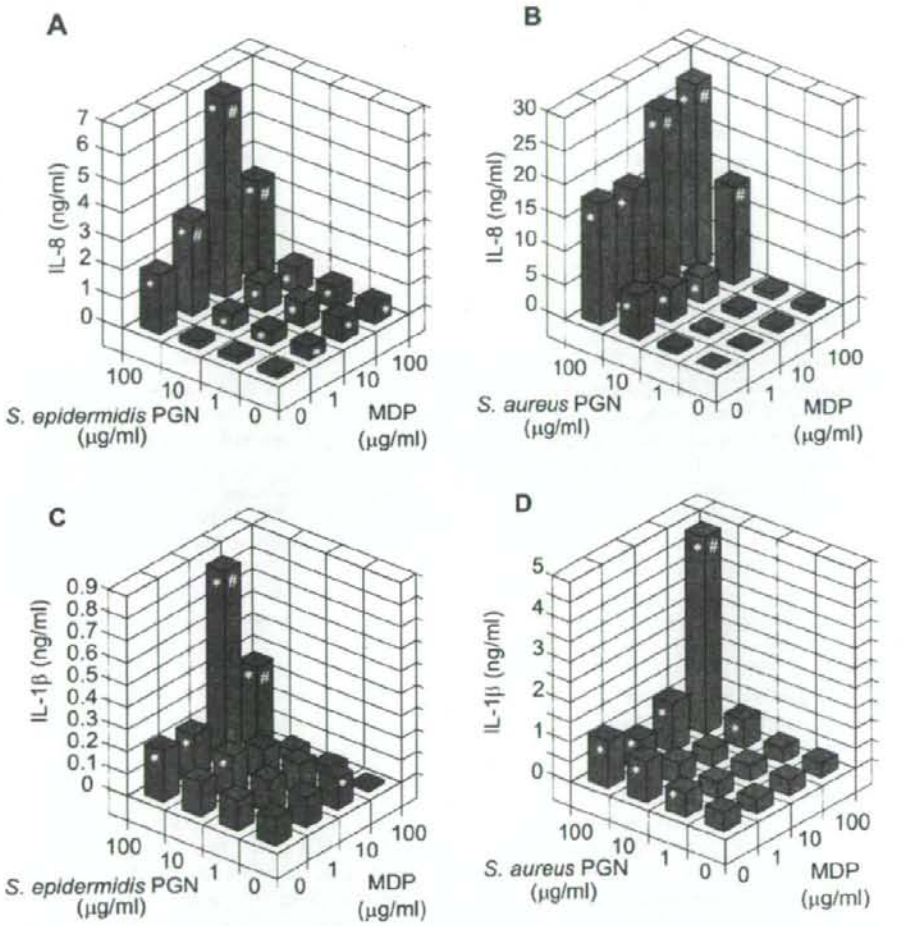

Fig. 6. Peptidoglycans and MDP induced the synergistic activation of THP-1 cells to induce pro-inflammatory cytokines. OCT-treated THP-1 cells were stimulated with PGNs prepared from *S. epidermidis* (A, C) or *S. aureus* (B, D) in combination with MDP at the indicated concentration for 24 h, and then IL-8 (A, B) and IL-1β (C, D) in the culture supernatants were measured by ELISA in triplicate. '# $P < 0.01$ versus respective control. Significant synergistic effects were detected in (A–D) by ANOVA, including an interaction term.

upon combinatory stimulation with TLR2 and NOD1 or NOD2 ligands, especially the latter. Furthermore, we showed that PGN fragments induced the production of IL-1β (Fig. 4). As IL-1β is synthesized as a pro-protein that is cleaved via caspase-1, the activation of the inflammasome or cleavage of caspase-1 is indispensable to induce IL-1β. In this context, Marinon *et al.*[33] reported that the MDP structure activated inflammasome via NALP3, resulting in the release of IL-1β. Further study is required to elucidate the mechanism to activate inflammasome and induce IL-1β by peptidoglycan-related preparations.

There are numerous examples of incorrect reports using contaminated bacterial compounds; therefore, we used chemically-defined preparations, especially chemically synthesized preparations, as far as possible. In this study, we used synthetic MDP and FSL-1 as reference monomer structures of peptidoglycan and lipoprotein, respectively; however, chemically synthesized preparations of polymer-type peptidoglycan, are not available at present. The SEPS used in this study is only available as a chemically-defined, water-soluble, peptidoglycan polymer prepared from purified peptidoglycan by enzymatic digestion using purified and defined enzyme

SALE endopeptidase.[22] As described above, amino acids different from PGNs were barely detected in SEPS and SEPS-M.[22,23] Furthermore, there were no essential differences in the constituent amino sugars and amino acids between SEPS and SEPS-M,[23] suggesting that TLR2-agonistic activity of SEPS should be attributable to the peptidoglycan structure, not to TLR2-agonistic contaminant such as lipoprotein[34] and lipoteichoic acid.[35,36] Recently, Zähringer *et al.*[37] suggested that the putative bacterial components so far reported as TLR2 agonists were due to contaminating, highly active, natural lipoproteins and/or lipopeptides; therefore, we treated SEPS and SEPS-M with lipoprotein lipase, which diminished or reduced activities of lipoproteins.[25,38] The treatment did not influence the activities of SEPS and SEPS-M, while the activity of reference lipopeptide FSL-1 was markedly reduced (Fig. 3), further suggesting that TLR2-agonistic activity of SEPS is not attributable to lipoprotein(s). Therefore, the present study reconfirmed the existence of TLR2-agonistic water-soluble PGN fragments, which were previously reported by Yoshimura *et al.*[18] and Dziarski and Gupta.[19] These findings strongly suggested that a repeating structure is required for PGNs to sense TLR2, namely, the pattern recognition of PGNs by TLR2 might depend on the repeating structure of PGNs. In this context, peptidoglycan and cell wall fractions also possess a polymer structure carrying the intact glycan chain, and are, therefore, capable of activating TLR2; however, water-insoluble preparations could not reach and activate intracellular NOD2. TLR2 activation by cell wall and peptidoglycan preparations resulted in only weak IL-8 secretion (Figs 2, 4, and 5). A similar recognition system was reported in an alternative pathway of the complement system.[39] Cell walls, peptidoglycans and SEPS powerfully activated the complement system, while SEPS-M and MDP were scarcely active in this respect. Concerning SEPS-M, weak TLR2-agonistic activity remained, which possibly synergistically activate THP-1 cells (Figs 2 and 5), although another human monocytic cell line, U-937, was non-responsive to the TLR2-agonistic activity of SEPS-M (Fig. 4). It is possible that the weak TLR2-agonistic activity remaining in SEPS-M is attributable to an unknown TLR2-agonistic contaminant other than lipoprotein/lipopeptide and the same contaminant possibly also exists in SEPS, although this activity could explain only a small part of the TLR2-agonistic activity of SEPS.

It must be noted, however, that the present study is the first to describe clear synergism between TLR2 and NOD2 signaling in the same molecule. In this context, Myhre *et al.*[40] reported the importance of the glycan chain for PGNs to induce hepatic and renal injuries in mice and cytokine release in the human whole blood culture system using PGNs of *S. aureus* (L-Lys-type), *Bacillus subtilis* (*meso*-DAP-type), and *Curtobacterium flaccumfaciens* (L-homoserine type), although they did not consider the possible involvement of either TLR2 or NOD1/2 in their activities. Conversely, they clearly described in the human whole blood culture system that synergism was not observed between monomer-type PGN prepared enzymatically from *S. aureus* PGNs and intact *S. aureus* PGNs. In this context, Wolfert *et al.*[41] reported the clear synergistic effect of MDP and *S. aureus* PGNs in human Mono Mac 6 cells to release TNF-α, although they claimed that the phenomenon might be merely apparent. Our findings should be supported by studies using chemically-synthesized peptidoglycan polymers in the future.

Various bacteria have PGN-lytic enzymes.[42] Host cells are also suggested to cleave bacterial PGNs;[43–45] therefore, there is a good possibility of the synergistic activation of host cells by TLR2- and NOD2-agonistic water-soluble polymer-type PGN fragments like SEPS. Furthermore, the synergism between TLR2-agonistic intact PGNs and NOD2- and/or NOD1-agonistic enzymatic digests (as shown in Figure 5 in this study) is more consequent. In this context, a mammalian PGN-recognition protein, PGRP-L (PGLYRP-2)[46] was recently revealed to be *N*-acetylmuramyl-L-alanine amidase, which possibly produces NOD1-agonistic fragments from DAP-type PGN. Thus, synergism between intact DAP-type PGNs and their NOD1-agonistic fragments might also usually occur. PGNs might be the most important structures of bacteria, because PGNs are recognized by three types of innate immune molecules – TLR2, NOD1/2 and PGRPs.

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Alendronate augments interleukin-1β release from macrophages infected with periodontal pathogenic bacteria through activation of caspase-1

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ABSTRACT

Nitrogen-containing bisphosphonates (NBPs) are anti-bone-resorptive drugs with inflammatory side effects that include osteomyelitis and osteonecrosis of the jaw. Oral bacteria have been considered to be a trigger for these NBP-associated jaw bone diseases. The present study examined the effects of alendronate (a typical NBP) and clodronate (a non-NBP) on the production of proinflammatory cytokines by macrophages infected with *Porphyromonas gingivalis* and *Tannerella forsythia*, which are important pathogens of periodontal diseases. Pretreatment with alendronate augmented IL-1β, but not TNFα, production by macrophages infected with *P. gingivalis* or *T. forsythia*. This augmentation of IL-1β production was inhibited by clodronate. Furthermore, caspase-1, a promoter of IL-1β production, was activated by treatment with alendronate, and caspase-1 inhibitor reduced the production of IL-1β induced by alendronate and *P. gingivalis*. These results suggest that NBPs augment periodontal pathogenic bacteria-induced IL-1β release via caspase-1 activation, and this phenomenon may contribute to the development of NBP-associated inflammatory side effects including jaw osteomyelitis. Co-treatment with clodronate may prevent and/or reduce these inflammatory effects induced by NBPs.

Introduction

Bisphosphonates (BPs) are synthetic pyrophosphate analogues that can inhibit bone resorption and are clinically used in the treatment of various diseases associated with increased bone resorption, such as Paget's disease, hypercalcemia of malignancy, osteoporosis, and tumor-induced bone-related diseases (Fleisch, 1991a, 1991b; Mundy, 1999; Rogers et al., 2000; Lipton, 2004). BPs can be divided into two different pharmacological classes: nitrogen-containing bisphosphonates (NBPs) and non-nitrogen-containing bisphosphonates (non-NBPs). NBPs are more potent in inhibiting bone resorption, but have undesirable inflammatory side effects, such as fever, increase in acute-phase proteins, gastrointestinal disturbance, and ophthalmic inflammation (Adami et al., 1987; Siris, 1993; Macàrol and Frauenfelder, 1994; Sauty et al., 1996; Fleisch, 1997; Thiébaud et al., 1997). Recently, jaw osteomyelitis and osteonecrosis have been reported as additional complications of NBP treatment (Ruggiero et al., 2004; Bagan et al., 2005; Yarom et al., 2007). Oral bacteria and/or stimulation by oral surgery have been suggested to be triggers and/or promoters of these NBP-associated jaw bone diseases (Hellstein and Marek, 2005).

Porphyromonas gingivalis is a black-pigmented anaerobic gram-negative bacterium that is an important etiologic agent of human chronic periodontitis (Lamont and Jenkinson, 1998; Craig et al. 2003). *Tannerella forsythia*, a gram-negative bacterium, has recently emerged as an important periodontal pathogen (Grossi et al., 1994, 1995; Tanner et al., 1998). Host responses to these periodontal bacteria are thought to cause the destruction of periodontal tissues and resorption of alveolar bone, and may be involved in the development of aspiration pneumonia and atherosclerosis (Darveau et al., 1997; Scannapieco, 1999; Chun et al., 2005). Macrophages play an essential role in these diseases, in part through their ability to secrete proinflammatory cytokines, including interleukin-1β (IL-1β) and tumor necrosis factor α (TNFα), in response to the bacteria and their cell wall components (Stoufi et al., 1987; Suzuki et al., 1997; Hajishengallis et at., 2002).

IL-1β is a key mediator of host immune responses and plays an important role in the development of inflammatory disease and tissue destruction (Dinarello, 1996; Gauldie et al., 2007). In response to proinflammatory stimuli, including pathogenic bacteria, the IL-1β precursor is induced in the cytosol of monocytes and macrophages (Dinarello, 2006). Secretion of IL-1β by macrophages depends on caspase-1, a protease that converts the IL-1β precursor to the mature and biologically active cytokine (Thornberry et al., 1992). Caspase-1 is initially expressed as an inactive zymogen and is activated by self-cleavage within a multiple adaptor complex termed the inflammasome (Martinon et al., 2002).

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We previously reported that alendronate (a typical NBP) and lipopolysaccharide (LPS) mutually augment inflammatory reactions in mice, including IL-1β production, while co-administration of clodronate (a non-NBP) with alendronate inhibited these inflammatory reactions (Deng et al., 2006). To elucidate the relationship between oral bacteria and BP-associated jaw osteomyelitis and osteonecrosis, we investigated the effects of BPs on proinflammatory cytokines produced by macrophages in response to *P. gingivalis* or *T. forsythia* infection. In addition, we examined whether caspase-1 is activated by alendronate.

Materials and methods

Reagents. Alendronate and clodronate (LKT Laboratories, Inc., St. Paul, MN, USA) were dissolved in saline and adjusted to pH 7 with NaOH. Pam_3Cys-Ser-$(Lys)_4$ (Pam_3CSK_4, Peptide Institute, Inc., Osaka, Japan) was dissolved in endotoxin-free water. Lipid A compound 506 (Peptide Institute), caspase-1 inhibitor (Ac-YVAD-CHO) (Alexis Biochemicals, Lausen, Switzerland) and staurosporine (Cell Signaling Technology, Inc., Danvers, MA, USA) were dissolved in dimethylsulfoxide (DMSO). All reagents were diluted in RPMI 1640 medium (Sigma, St. Louis, MO, USA) before use.

Cell culture and bacteria. Murine macrophage-like J774.1 cells were obtained from Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). The cells were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (FBS; GIBCO, Carlsbad, CA, USA), penicillin (100 U/ml) and streptomycin (100 μg/ml) in an incubator at 37 °C and 5% CO_2. *P. gingivalis* ATCC 33277 was anaerobically grown in GAM broth supplemented with hemin (5 μg/ml) and menadione (1 μg/ml) at 37 °C. *T. forsythia* ATCC 43037 was anaerobically grown in brain heart infusion broth (GIBCO) containing 5 μg/ml hemin, 1 μg/ml menadione, 0.001% *N*-acetylmuramic acid (Sigma), and 5% FBS at 37 °C.

Measurement of cytokines. Adherent J774.1 cells (2×10^5 cells/well) were pretreated with or without alendronate or clodronate for 24 h and washed with serum-free RPMI 1640 twice. Cells were then incubated with or without live *P. gingivalis* or *T. forsythia*, or stimulated with Pam_3CSK_4 or Lipid A in 96-well flat-bottomed plates (Falcon, Franklin Lakes, NJ, USA) for an additional 24 h. For caspase-1 inhibition assays, J774.1 cells were treated with caspase-1 inhibitor (Ac-YVAD-CHO) for 2 h prior to *P. gingivalis* infection. Levels of secreted mouse IL-1β and TNFα in culture supernatants were measured by ELISA (eBioscience, San Diego, CA, USA).

Caspase-1 activation assay. To quantify activation of caspase-1 in J774.1 cells by flow cytometry, a FLICA (fluorochrome inhibitor of caspases) assay using the caspase-1 staining reagent carboxy-fluorescein-YVAD-fmk (FAM-YVAD-fmk) was used according to the manufacturer's instructions (Immunochemistry Technologies, LLC, Bloomington, MN, USA). J774.1 cells (1.5×10^7 cells in T-25 flasks) were treated with alendronate or clodronate for 24 h. Cells were washed with serum-free RPMI 1640 twice, infected with *P. gingivalis* for another 24 h, collected, and incubated with caspase-1 FLICA staining reagent. The cells were washed and fixed according to the manufacturer's instructions and analyzed by flow cytometry for caspase-1 activity.

Data analysis. All experiments in this study were repeated at least twice to confirm the reproducibility of the results. The data shown are representative results. Experimental values are given as the mean±SD of triplicate assays. Statistical analysis was performed with one-way analysis of variance using the Bonferroni's or Dunnett's method and $P<0.05$ was considered statistically significant.

Results

Pretreatment with alendronate augments IL-1β production by J774.1 cells infected with P. gingivalis or T. forsythia

We previously demonstrated that pretreatment with alendronate, a nitrogen-containing bisphosphonate, augmented LPS-induced IL-1β production *in vivo* (Sugawara et al., 1998; Deng et al., 2006). In the present study, a mouse macrophage-like cell line, J774.1, was used to examine the effects of alendronate treatment. In uninfected cells,

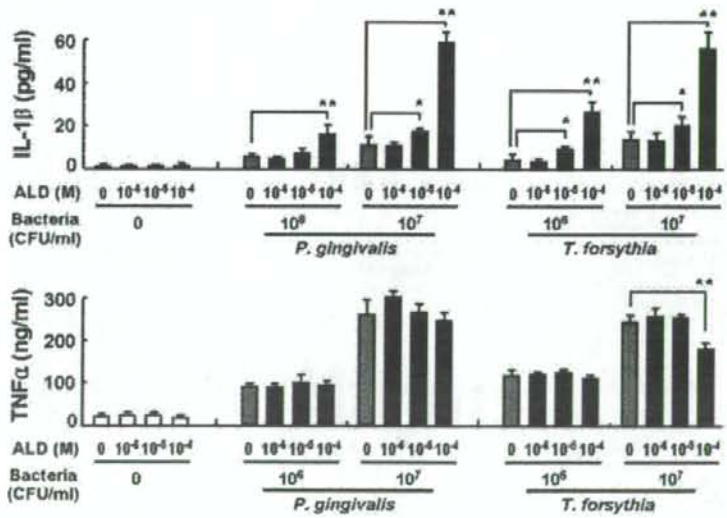

Fig. 1. Effects of pretreatment with alendronate on proinflammatory cytokine production by J774.1 cells infected with *P. gingivalis* (*P.g*) or *T. forsythia* (*T.f*). J774.1 cells were incubated in medium with or without alendronate (ALD) (10^{-6}, 10^{-5}, or 10^{-4} M) for 24 h. Cells were then washed with medium, incubated with or without *Pg* or *T.f* (1×10^6 or 1×10^7 CFU/ml) for 24 h, and culture supernatants were collected. Results are presented as the mean±SD of triplicate cultures. *$P<0.05$ and **$P<0.01$.

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alendronate treatment alone did not induce IL-1β or TNFα release (Fig. 1). However, pretreatment with alendronate promoted IL-1β, but not TNFα, release induced by *P. gingivalis* and *T. forsythia* in a dose-dependent manner.

Next, J774.1 cells were incubated with *P. gingivalis* or *T. forsythia*, which was followed by treatment with alendronate. Alendronate treatment post-infection did not augment bacteria-induced IL-1β production, nor did it affect TNFα production (Fig. 2). These data indicate that IL-1β production induced by *P. gingivalis* and *T. forsythia* is augmented by treatment with alendronate prior to, but not after infection, suggesting that alendronate may act on molecules involved in IL-1β production in J774.1 cells.

The time course of cytokine production by alendronate-treated, *P. gingivalis*-stimulated J774.1 cells showed that alendronate promoted IL-1β production after 9 h of exposure to *P. gingivalis* (Fig. 3). TNFα production was slightly but significantly reduced by alendronate.

IL-10, an anti-inflammatory cytokine, has been reported to inhibit proinflammatory cytokine production (Fiorentino et al., 1991; de Waal Malefyt et al., 1991; Crabtree et al., 2001). However, IL-10 production was not altered by alendronate or *P. gingivalis* (Fig. 3), suggesting that IL-10 may be not involved in the regulation of IL-1β and TNFα production induced by alendronate and/or *P. gingivalis*.

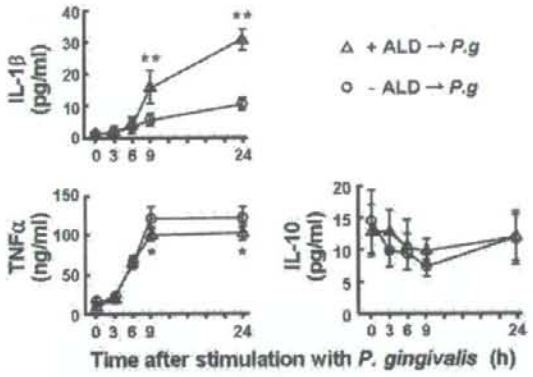

Fig. 3. Time course of *P. gingivalis* (*P.g*)-induced cytokine production by J774.1 cells pretreated with alendronate. J774.1 cells were incubated with or without alendronate (ALD) (10^{-4} M) for 24 h. Cells were washed with medium and incubated with *P.g* (1×10^7 CFU/ml). At the indicated time points, culture supernatants were collected and IL-1β, TNFα, and IL-10 levels were measured. Results are presented as the mean ± SD of triplicate cultures. *$P<0.05$ and **$P<0.01$ compared with −ALD → *P.g*.

Pretreatment with alendronate augments IL-1β production stimulated by Toll-like receptor (TLR) ligands

To examine the effects of alendronate on TLR-induced IL-1β production, J774.1 cells were treated with alendronate, then incubated with a TLR2/1 ligand Pam_3CSK_4 or a TLR4 ligand lipid A. IL-1β production stimulated by these bacterial components were also promoted by alendronate-pretreatment (Fig. 4). These data suggest that the augmenting effect of alendronate on IL-1β production may be common among bacterial species.

Clodronate inhibits the augmenting effect of alendronate on IL-1β production

Unlike alendronate, clodronate is a non-nitrogen-containing bisphosphonate. We previously reported that clodronate inhibits alendronate-induced inflammatory reactions (Deng et al., 2006). To examine the effect of clodronate on IL-1β production by bacterially infected cells, J774.1 cells were treated with alendronate and/or clodronate, followed by incubation with *P. gingivalis* or *T. forsythia*. While clodronate did not have an effect on IL-1β production by uninfected cells, pretreatment with clodronate markedly inhibited IL-1β production induced by alendronate and bacterial infection (Fig. 5).

Caspase-1 is activated by alendronate treatment of J774.1 cells

Since caspase-1 is an IL-1β converting enzyme required for processing the inactive IL-1β precursor to the active form in macrophages (Kuida et al., 1995), it is possible that caspase-1 is involved in the augmenting effect of alendronate on IL-1β production.

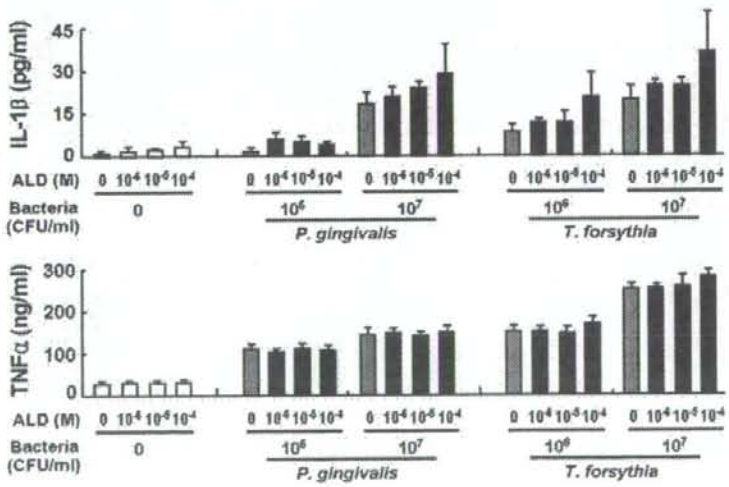

Fig. 2. Effects of alendronate treatment of *P. gingivalis* (*P.g*)- or *T. forsythia* (*T.f*)-infected J774.1 cells on proinflammatory cytokine production. J774.1 cells were incubated with medium alone, *P.g*, or *T.f* (1×10^6 or 1×10^7 CFU/ml) for 24 h. Alendronate (ALD) (10^{-6}, 10^{-5}, or 10^{-4} M) was added to the culture medium and culture supernatants were collected after 24 h. Results are presented as the mean ± SD of triplicate cultures.

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To test this hypothesis, caspase-1 activity in J774.1 cells treated with alendronate and/or clodronate for 24 h was analyzed by flow cytometry. Caspase-1 in J774.1 cells was activated by incubation with alendronate for 24 h (Fig. 6A). The alendronate-induced caspase-1 activation was markedly increased after an additional 24 h incubation in alendronate-free medium (Fig. 6B). These data suggest that an alendronate-induced signal is active even in the absence of alendronate in the additional incubation period. The alendronate-induced activation of caspase-1 was further increased in the presence of *P. gingivalis* (Fig. 6C). In these experiments, clodronate reduced the alendronate-induced activation of caspase-1, although its inhibitory effect was weak (Figs. 6A–C). These results indicate that alendronate alone or in combination with *P. gingivalis* infection can induce the activation of caspase-1, which is marginally inhibited by clodronate. The fluorescence intensity of staurosporine-stimulated cells and unstained cells were used as positive and negative controls, respectively (Fig. 6D).

Alendronate-induced IL-1β production requires caspase-1 activity

To examine the role of caspase-1 in alendronate-induced IL-1β production, alendronate-treated J774.1 cells were incubated with various concentrations of a caspase-1 inhibitor, Ac-YVAD-CHO, prior to *P. gingivalis* stimulation. Ac-YVAD-CHO inhibited IL-1β production

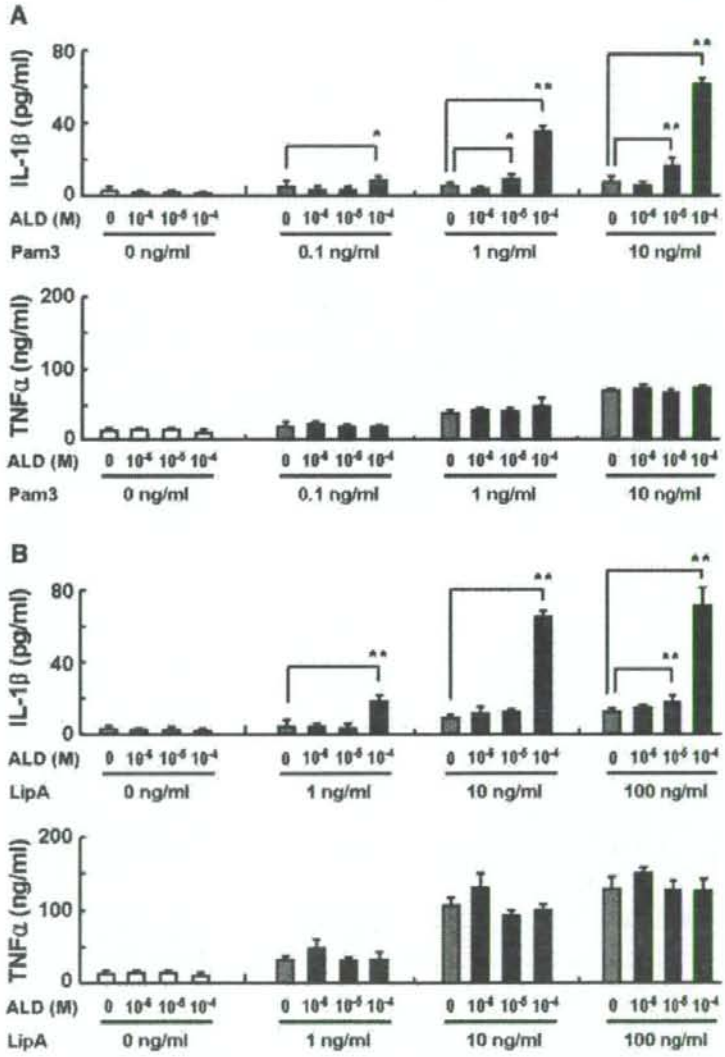

Fig. 4. Effects of alendronate on proinflammatory cytokine production by J774.1 cells stimulated with TLR ligands. J774.1 cells were incubated with or without alendronate (ALD) (10^{-6}, 10^{-5}, or 10^{-4} M) for 24 h, washed, then stimulated with Pam_3CSK_4 (Pam3) (0.1, 1 or 10 ng/ml) (A) or lipid A (LipA) (1, 10 or 100 ng/ml) (B). IL-1β and TNFα levels in the culture supernatants were measured 24 h later. Results are presented as the mean ± SD of triplicate cultures. *$P<0.05$ and **$P<0.01$.

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in a dose-dependent manner (Fig. 7). These results demonstrate that alendronate augments *P. gingivalis*-induced IL-1β production via the activation of caspase-1.

Discussion

NBPs inhibit bone resorption by causing osteoclast inactivation and/or apoptosis (Fisher et al., 1999), and can act on macrophages (Makkonen et al., 1999; Frith and Rogers, 2003), osteoblasts (Plotkin et al., 1999) and γδT cells (Kunzmann et al., 1999, 2000). As treatment with alendronate prior to, but not after, bacterial infection augmented bacteria-induced IL-1β production by J774.1 cells (Figs. 1–3), it is possible that alendronate acts on macrophages to regulate molecules involved in IL-1β production. *P. gingivalis* and TLR4 ligands have been shown to induce proIL-1β protein synthesis and caspase-1 activation (Ogawa and Uchida, 1996; Taxman et al., 2006). In this study, treatment with alendronate induced caspase-1 activation in J774.1 cells (Fig. 6). Furthermore, the IL-1β increase induced by alendronate and *P. gingivalis* was blunted by a caspase-1 inhibitor (Fig. 7), suggesting that alendronate augments *P. gingivalis*-induced IL-1β production via caspase-1 activation. However, the inhibitory effect of the caspase-1 inhibitor on IL-1β production was not complete, indicating that other factors may be involved in the augmenting effect of alendronate on IL-1β production. Our results suggest that NBP-augmented IL-1β production in response to periodontal bacteria may contribute to the development of NBP-associated jaw osteomyelitis and other inflammatory side effects. Moreover, NBPs deposit for a significantly long time in the spleen and liver (Mönkkönen et al., 1990) and induce inflammatory reactions including IL-1β production in various cell-types, organs, tissues, and blood (Sugawara et al., 1998; Endo et al., 1993, 1999; Deng et al., 2006, 2007). *P. gingivalis*-associated aspiration pneumonia and atherosclerosis (Darveau et al., 1997; Scannapieco, 1999; Chun et al., 2005) may potentially be aggravated by NBP-treatment via an increase in IL-1β production. Ballester et al. (2007) reported that oral treatment with alendronate reduced IL-1β mRNA levels in a rat model of trinitrobenzenesulfonic acid-induced colitis. However, intraperitoneal administration of alendronate resulted in increased loss of body weight, spreading of necrosis in the colon, and failure to inhibit the inflammatory responses in colitic rats. Oral bioavailability of alendronate, as with other bisphosphonates, is approximately 1% (Lin et al., 1994; Porras et al., 1999). Therefore, low-dose alendronate may show an anti-inflammatory effect, while high doses may result in toxicity and intensify inflammatory reactions in rodents.

NOD-like receptors (NLRs) are intracellular pattern-recognition receptors that detect microbial components in the cytosol (Kanneganti et al., 2007; Franchi et al., 2008). Members of the NLR family, such as NALP1, NALP3, and Ipaf, promote the assembly of inflammasomes and subsequently activate caspase-1 (Mariathasan et al., 2004; Mariathasan and Monack, 2007). NOD2 associates with NALP1 to form a complex that activates caspase-1 in response to muramyl dipeptide (Hsu et al., 2008). As alendronate is internalized by J774.1 cells (Frith and Rogers, 2003), it is possible that alendronate-induced caspase-1 activation is associated with NLRs. Further studies are needed to elucidate the mechanisms of caspase-1 activation by alendronate. In contrast, TNFα production did not increase, but rather slightly decreased with alendronate treatment in J774.1 cells (Figs. 1–4). Furthermore, we previously reported that LPS-induced TNFα production was suppressed in alendronate-pretreated mice (Deng et al., 2006). TNFα production seems to be not reduced by IL-10, as IL-10 production was not increased in this experiment (Fig. 3).

The NBP-induced inflammatory actions can be largely suppressed by clodronate at a dose equivalent to that of alendronate used in mouse models (Endo et al., 1999; Deng et al., 2006) and macrophage-like J774.1 cells (Fig. 5). However, the mechanism of inhibition is unclear. In the present study, the effects of clodronate on caspase-1 activation induced by alendronate or alendronate and bacteria were investigated. However, the inhibitory effect of clodronate on caspase-1

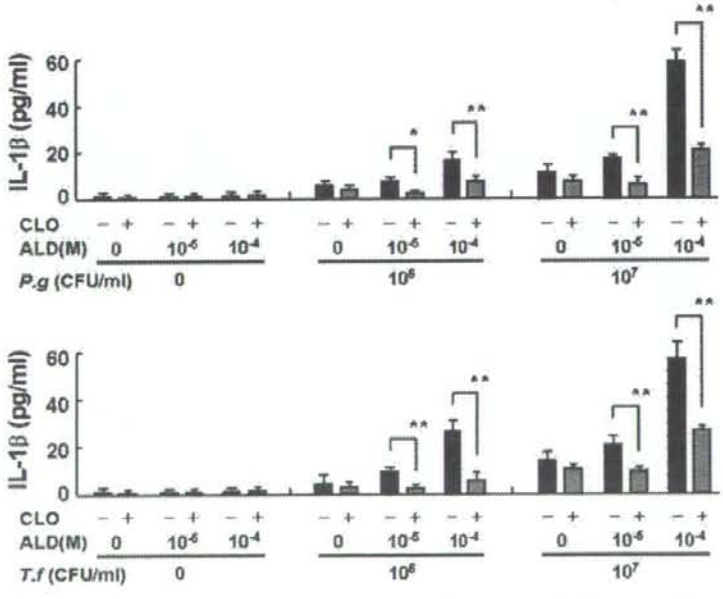

Fig. 5. Inhibitory effects of clodronate on the augmenting effect of alendronate on IL-1β production induced by *P. gingivalis* (*P.g*) and *T. forsythia* (*T.f*). J774.1 cells were treated with alendronate (ALD) (10^{-5} or 10^{-4} M) and/or clodronate (CLO) (10^{-4} M) for 24 h, washed, and further incubated with *P.g* or *T.f* (1×10^{6} or 1×10^{7} CFU/ml). After 24 h, IL-1β levels in the culture supernatants were measured. Results are presented as the mean ± SD of triplicate cultures. *$P<0.05$ and **$P<0.01$.

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activation appeared too weak (Fig. 6) to explain its potent inhibition of IL-1β production (Fig. 5). It has been proposed that clodronate might compete with NBPs for cellular uptake via a membrane-bound transport protein in osteoclasts, J774.1 cells, and human peripheral blood γδT cells, although no such protein has yet been identified (Frith and Rogers, 2003; Thompson and Rogers, 2004). However, the *in vitro* competitive effect of clodronate is very weak, reaching significance only after a long period of culture (8 h or more) in the presence of 40-fold more clodronate than that of an NBP. Makkaonen et al. (1999) reported that both clodronate and its metabolite inhibited LPS-stimulated binding of NF-κB to DNA in macrophages. However, this transcription factor positively regulates proIL-1β mRNA and inhibits caspase-1 activation (Greten et al., 2007). Thus, the mechanism underlying the *in vitro* inhibitory effect of clodronate on inflammatory reactions induced by NBPs is complex and may involve various factors, including the interaction of several IL-1β-associated molecules. In any event, co-administration of clodronate and an NBP may contribute to prevent and/or reduce NBP-induced inflammatory side effects including jaw osteomyelitis.

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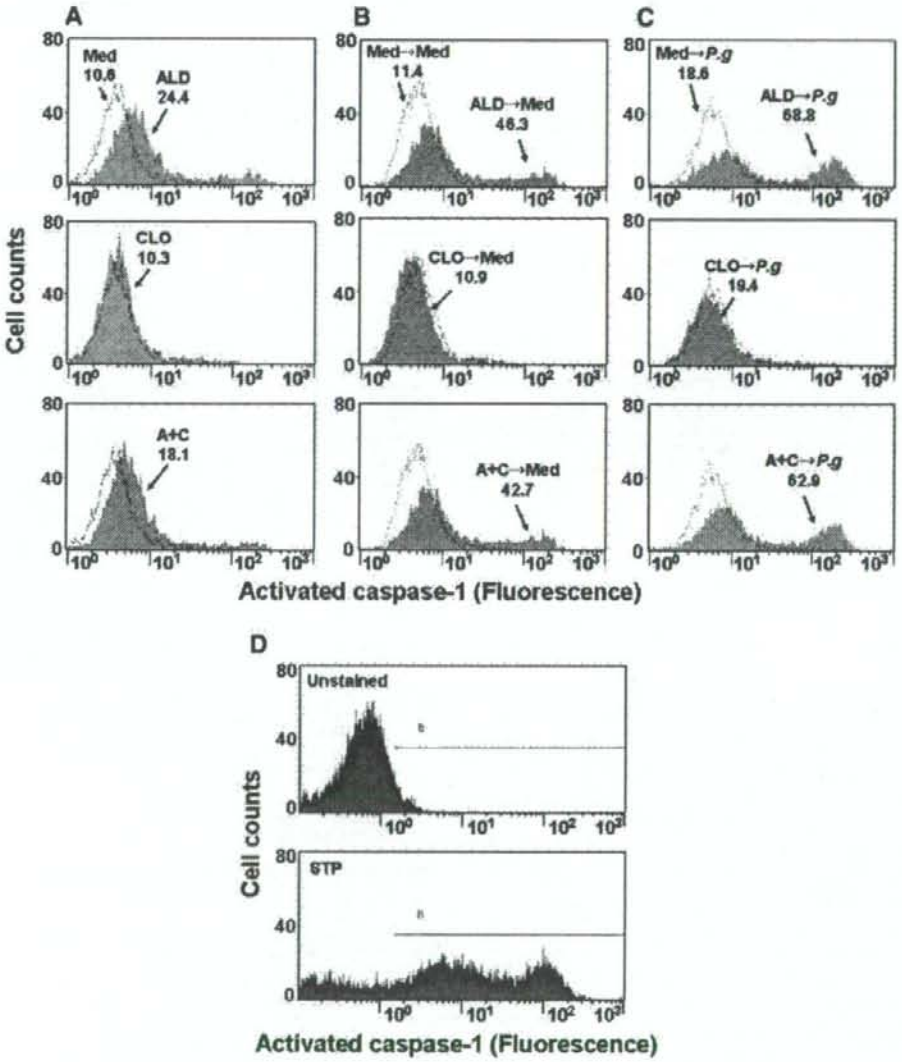

Fig. 6. Caspase-1 activation induced by alendronate in J774.1 cells. (A) Cells were incubated with medium (Med) alone or with alendronate (ALD) (10^{-4} M) and/or clodronate (CLO) (10^{-4} M) for 24 h. The cells treated with ALD and/or CLO were washed and incubated for an additional 24 h with Med alone (B) or with *P. gingivalis* (*P.g*) (C). The cells were incubated with staurosporine (STP) (1 μM) for 24 h (D). J774.1 cells were stained with or without FAM-YVAD-fmk and caspase-1 activity was analyzed by flow cytometry. Values indicated in the histograms represent the mean fluorescence intensity of cells. The results shown are representative of two independent experiments.

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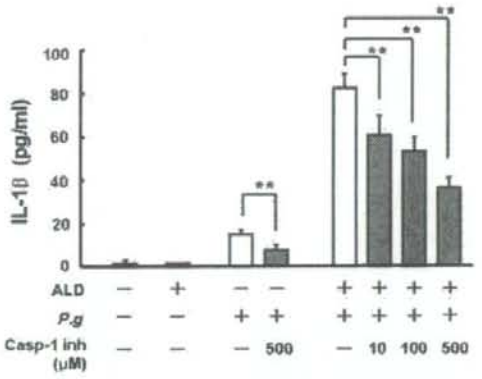

Fig. 7. Effects of caspase-1 inhibitor on alendronate-induced IL-1β production. J774.1 cells were incubated in medium with or without alendronate (*ALD*) (10^{-4} M) for 24 h. Cells were then washed with medium and incubated for an additional 24 h with *P. gingivalis* (*P.g*) (1×10^7 CFU/ml). Prior to *P.g* infection, caspase-1 inhibitor (Ac-YVAD-CHO) was added to the culture medium at concentrations ranging from 10 to 500 μM. IL-1β levels in the culture supernatants were measured by ELISA. Results are presented as the mean±SD of triplicate cultures.

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Necrotic Actions of Nitrogen-Containing Bisphosphonates (NBPs) and Their Inhibition by Clodronate (a non-NBP) in Mice: Potential for Utilization of Clodronate as a Combination Drug with an NBP

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Running title: anti-necrotic effects of clodronate

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Abstract: Nitrogen-containing bisphosphonates (NBPs) exhibit powerful anti-bone-resorptive effects (ABREs) via inhibition of farnesyl pyrophosphate synthase during cholesterol biosynthesis. Clinical applications have disclosed an unexpected side effect, osteonecrosis of jaw bones (ONJ), and although thousands of cases have been documented in the last few years the mechanism remains unclear. Since NBPs accumulate in bone-hydroxyapatite, more ONJ cases may come to light if NBPs continue to be used as now. We previously reported that in mice, systemic (intraperitoneal) injection of clodronate (a non-NBP) prevents the inflammatory effects of NBPs. Here, we examined in mice the local necrotic actions of various NBPs and the antinecrotic effects of clodronate. A single subcutaneous injection of an NBP into the ear pinna induced necrosis at the injection site (relative potencies of necrotic actions of NBPs: zoledronate >> pamidronate ≥ alendronate > risedronate), while non-NBPs lacked this effect. Clodronate, when injected together with an NBP, reduced or prevented the necrosis induced by that NBP, but not its ABRE. Clodronate reduced the amount of each NBP retained within tissues. These results, together with those of previous studies, suggest that (i) clodronate inhibits the inflammatory and necrotic actions of NBPs by inhibiting their incorporation into cells related to inflammation and/or necrosis, (ii) clodronate could be useful as a combination drug with NBPs for preventing their necrotic actions while retaining their ABREs, and (iii) clodronate could also be useful as a substitution drug for NBPs in patients at risk of ONJ.

Bisphosphonates (BPs) have a non-hydrolysable P-C-P bond and bind to bone hydroxyapatite (BHA), and their ingestion by osteoclasts results in anti-bone-resorptive effects (ABREs) (Fig. 1). Among the BPs, nitrogen-containing BPs (NBPs) have ABREs that are much more powerful than those of non-nitrogen-containing BPs (non-NBPs). NBPs exert their ABREs via inhibition of farnesyl pyrophosphate (FPP) synthase during cholesterol biosynthesis, while non-NBPs exert theirs via their conversion into cytotoxic analogues of ATP [1-3]. BPs are important drugs for use against diseases involving enhanced bone resorption (osteoporosis, tumoral osteolysis, tumoral hypercalcemia,

osteogenesis imperfecta, Paget's disease, and rheumatoid arthritis). Recent studies have also led to NBPs being used against bone metastases [4, 5]. Currently, BPs, especially NBPs, are in use globally in millions of patients with these diseases.

However, most NBPs have inflammatory side effects (such as fever, increase in acute-phase proteins, gastrointestinal disturbance, and ophthalmic inflammation) [6-11]. Febrile reactions occur in many patients treated with intravenous NBPs for the first time, and even in significant numbers of patients treated with oral NBPs [10, 12, 13]. A serious influenza-like reaction has been reported in children with osteogenesis imperfecta [14]. In addition, unexpectedly, clinical applications have recently disclosed a shocking side effect, the osteonecrosis of jaw bones (ONJ) and their subsequent exposure [15-18]. Indeed, in only a few years thousands of ONJ cases have already been documented [19]. Although oral bacteria and/or dental treatments are thought to trigger or promote this side effect [20, 21], the precise mechanism underlying it remains unclear [22]. It should also be noted that since NBPs accumulate in bones upon repeated administration, more ONJ cases may come to light, if NBPs continue to be used as they are now. This highlights the urgent need for strategies for their safe use, and indeed some are already in place [23].

In mice, a single intraperitoneal injection of an NBP induces a variety of inflammatory reactions (including a prolonged induction of the histamine-forming enzyme, pleural exudate, increased granulocytic cells, and splenomegaly) together with changes in hematopoiesis [24, 25]. This suggests that NBPs may affect the functions of a number of cell types in certain situations. Moreover, a mutual augmentation of inflammatory reactions is seen with NBPs and lipopolysaccharide (LPS, a cell-surface constituent of gram negative bacteria) [26-28]. A very interesting finding was that clodronate (a non-NBP) can reduce or prevent the inflammatory actions of NBPs in mice [29]. In terms of this protective effect, clodronate is superior both to aspirin (a non-steroidal anti-inflammatory drug) and to dexamethasone (a synthetic anti-inflammatory steroid) [30]. Clodronate can also prevent the mutual augmentation of inflammation between LPS and NBPs [28]. However, it is not clear how clodronate inhibits the inflammatory actions of NBPs. It has been reported that a significant amount of at least some NBPs (pamidronate and alendronate) is retained for a long time (several days) in soft tissues in mice or rats (liver, lung, and spleen), whereas clodronate is rapidly excreted [31, 32]. Thus, studying the pharmacological kinetics of NBPs may help clarify the mechanisms underlying the protective effect of clodronate.

Having considered the above background, we performed the present study to examine in mice (i) the necrotic effects occurring after a single subcutaneous injection of NBPs into ear pinnas, (ii) the effects of clodronate on such NBP-induced necrosis and on the ABREs of NBPs, and (iii) the effect of clodronate on the retention of NBPs in various tissues (ear pinna, liver, lung, and spleen).

Materials

Mice. BALB/c mice were bred in our laboratory. All experiments complied with the Guidelines for Care and Use of Laboratory Animals in Tohoku University.

Reagents. Alendronate, pamidronate, and risedronate were purchased from LKT Laboratories, Inc. (St. Paul, MN, USA). Zoledronate and clodronate were from Toronto Research Chemicals Inc. (North York, ON, Canada) and Sigma (St. Louis, MO, USA), respectively. The above drugs were dissolved in sterile saline, with the pH of the solutions being adjusted to 7 with NaOH. Experimental protocols are described in the text or in the legend to the Figure relating to each experiment.

Methods

Three experiments (I, II, and III) were performed.

I. Inflammatory and necrotic actions of NBPs. Female mice (6-8 weeks of age) were anesthetized with ethyl ether, and an NBP solution was injected subcutaneously into both the right and the left pinna (inside) near the root of the ear (20 μl each ear) (3 mice/group) (Fig. 2). The concentrations used are indicated in the relevant experiments. The inflammatory and necrotic actions of NBPs were evaluated daily as described below. All experiments were terminated on finished day 7.
(a) Assessment of inflammation: The length (L) and width (W) of the area of inflammation at the backside of the ear (detectable as a red area) were recorded, and L x W (mm^2) was used as an indicator of inflammation.
(b) Assessment of necrosis: After maximum inflammation (estimated as described above) had been attained, the center of the inflammatory site became necrotic [detectable as a change of color from red to dark brown (or black) or a tissue defect]. At the start of the necrosis, we stopped measuring inflammation, and in each group of mice we recorded the number of ears with and the number without necrosis [expressed as the incidence of necrosis (i.e., maximum incidence is 6 in a group of 3 mice).

II. Estimation of ABREs of NBPs. A clear sclerotic band (tentatively called the BP band) is detectable in tibias by radiography a few weeks after a single intraperitoneal injection of a BP into mice, reflecting an inhibition of bone-resorption [30, 33, 34]. Hence, we can estimate the ABREs of NBPs by using the BP band as a marker. Briefly, each NBP solution was intraperitoneally injected (0.2 ml/mouse) into young male mice (4-5 weeks old). The mice were decapitated two weeks later, and tibias were removed and subjected to X-ray analysis for the detection of the BP band. The detection of BP bands and their quantification were carried out as follows. Soft X-ray radiophotographs were made using SOFTEX and Fuji Industrial X-ray film, the conditions being 80 V, 1 mA, duration 55 sec [33]. BP bands were quantitatively analyzed using NIH Image software. In this analysis, "mean gray values" (average gray value of pixels within a selected band) were compared. In each experiment, the mean gray value obtained from a BP band in a given experimental group was subtracted with that obtained from corresponding areas of normal tibias (n = 3).

III. Effects of clodronate on NBP levels in tissues after NBP injection. Although the use of isotope-labeled NBPs may be useful for this purpose, such NBPs are not available commercially. BP band formation is dependent on the dose of the BP [30]. Hence, in the present study, we utilized the BP band as a way of evaluating the amounts of NBP present within in tissues. Briefly, three hours after injection of an NBP, clodronate, or the NBP plus clodronate into male mice (6-8 weeks old), tissues were taken (see legend to Fig. 7) and homogenized in 3.5 ml of 0.4 M $HClO_4$. When the above injection was made into the ear pinna, the ear alone was subjected to NBP analysis. When the injection was made intraperitoneally, the liver, spleen, and lung were subjected to NBP analysis. The homogenate of each tissue was subjected to centrifugation (2,000 *g* for 5 min at room temperature) and its supernatant fraction (3 ml) was recovered. Next, the supernatant was neutralized to around pH 7 with 2 M KOH while monitoring with a pH meter. After this neutralization, the volume of each sample was adjusted to 4 ml with saline. Then, the sample was again subjected to centrifugation under the above conditions to remove $KClO_4$,

and its supernatant fraction (tentatively called a "tissue extract") was intraperitoneally injected (0.5 ml/mouse) into young male mice (4-5 weeks old). Two weeks later, tibias were removed and subjected to analysis of the BP band (see above). Actual protocols are described in each experiment (see text or legends to individual figures).

Data analysis. Experimental values are given as mean ± standard deviation (SD). The statistical significance of the difference between two means was evaluated using a Bonferroni multiple-comparison test. *P* values less than 0.05 were considered to be significant.

Results

I. Inflammatory and necrotic actions of NBPs.

(1) Inflammatory and necrotic reactions in the ear.
First, we tested zoledronate (the NBP associated with the highest incidence of ONJ and the highest ABRE among the current NBPs). Zoledronate at 4 mM induced inflammation within 3 days after the injection, the reaction being detectable as redness around the injection site. Within 4-6 days of the injection, the inflammatory areas exhibited necrosis [detectable as a change of color from red to dark brown (or black), frequently resulting in a partial defect of the tissue]. In the following experiments, we recorded the area of inflammation and the incidence of necrosis as described in Methods.

Next, we compared the inflammatory and necrotic effects of pamidronate, alendronate, risedronate, and zoledronate (Fig. 2). Each of these NBPs induced inflammatory and necrotic effects in a dose-dependent manner, their relative potencies being zoledronate >> pamidronate ≥ alendronate > risedronate. The doses of these NBPs inducing necrosis were higher than those inducing inflammation. Indeed, inflammation without necrosis was seen in mice injected with 8 mM of Pam, 4 mM Ale, 4 mM Ris, or 1 mM Zol (Fig. 2), indicating that inflammation precedes necrosis. In these experiments, the recording of inflammation was halted when the inflammatory area became necrotic. In preliminary experiments, the necrotic response to zoledronate was similar between young (6-8 weeks old) and aged (8 months old) female mice and also between young male and young female mice (6-8 weeks old) (data not shown). It should be noted that two non-NBPs (clodronate and etidronate) had no apparent inflammatory and necrotic effects even at 100 mM (data not shown).
(2) Effects of clodronate on the inflammatory and necrotic actions of NBPs.
Fig. 3 shows the effects of clodronate on the inflammatory and necrotic actions of zoledronate, the most potent NBP at inducing necrosis. The inflammatory action of 4 mM zoledronate was inhibited by clodronate in a dose-dependent manner, with the inflammation being almost completely inhibited by 4 mM clodronate. Concerning the necrotic action of 4 mM zoledronate, this was inhibited by clodronate at 2 mM or more, with the inhibition being total at 4 mM or more.

II. Estimation of ABERs of NBPs

(1) ABREs of NBPs, as evaluated by BP band formation.
Various solutions of NBPs (zoledronate, risedronate, alendronate, or pamidronate) (2.5-160 μM) were intraperitoneally injected into young mice, and two weeks later the tibias were subjected to the detection of BP bands. All these NBPs produced BP bands in a dose-dependent manner (Fig. 4), while clodronate formed detectable BP bands at 1 mM,

but not at 0.25 mM (data not shown). These data indicate that the relative potencies with which these agents form BP bands are zoledronate >> risedronate ≥ alendronate > pamidronate >> clodronate. This rank order corresponds to that previously reported [1, 2] (Fig. 1).

(2) Effects of clodronate on the ABREs of NBPs.

As described above, clodronate strongly inhibited the inflammatory and necrotic actions of NBPs. This led us to wonder whether clodronate might also inhibit the ABREs of NBPs. Hence, we examined the effects of clodronate on BP band formation by NBPs. Intraperitoneal injection of 0.4 mM (ca. 4 μmol/kg) of a given NBP into young mice induced clear BP bands in the tibias, with the BP bands induced by zoledronate having the highest mean gray value and also being the boldest (Fig. 5). The rank order of potencies for the formation of BP bands was Zol >> Ris = Ale (as evaluated from the data in Fig. 4). Co-injection of clodronate (4 mM) with a given NBP led to BP band formation that was similar to (risedronate and zoledronate) or stronger than (alendronate) that induced by the respective NBP alone. These results suggest that clodronate, when combined with an NBP, does not impair the potent ABRE of the NBP. To confirm this, we used a second method to examine the effect of clodronate on the ABREs of NBPs. This time, 0.1 mM of a given NBP, either alone or in combination with a 100-times higher concentration of clodronate (10 mM), was injected intraperitoneally into mice. As shown in Fig. 6, no significant differences were observed in the tibial BP-bands between mice given an NBP alone or the same NBP + clodronate. These results indicate that clodronate, despite having inhibitory effects on the inflammatory and necrotic actions of NBPs, does not impair their ABREs.

III. Effects of clodronate on NBP levels in tissues after NBP injection.

Mönkkönen et al [31] reported that after intravenous injection into mice, alendronate (an NBP) reaches plateau levels in the liver, lung, and spleen within a few hours and is retained for several days, whereas clodronate (a non-NBP) is rapidly excreted via the kidney within 30 min and is not retained in these tissues. In the present study, we examined the effects of clodronate on the retention of an NBP in tissues at 3 h after its injection. First, zoledronate alone or zoledronate + clodronate was injected subcutaneously into ear pinnas, and the levels of zoledronate in the ears were evaluated as described in Methods (using the tibial BP band in other mice as a marker). As shown in Fig. 7A, the BP band formation induced by an extract of ears injected with zoledronate + clodronate was markedly reduced (vs. zoledronate alone), indicating that the clodronate had reduced the retention of zoledronate in the ear tissues. We previously reported that clodronate strongly inhibited the induction by alendronate of the histamine-forming enzyme (histidine decarboxylase) in the liver, spleen, and lung [29]. We therefore examined the effects of clodronate on the levels of zoledronate or alendronate present in these three tissues after their intraperitoneal injection. As shown in Figs. 7B and 7C, we could not detect BP band formation when clodronate was combined with zoledronate or alendronate, indicating that clodronate inhibited the retentions of zoledronate and alendronate in these tissues. These results suggest that clodronate may inhibit the incorporation of NBPs into cells in various tissues.

Discussion

Summary of the findings made in the present study.

We examined the necrotic actions of NBPs and the effects of clodronate (a non-NBP) on

these using a mouse model. A single subcutaneous injection of an NBP into the ear pinna induced necrosis, as well as inflammation, at the injection site. The relative potencies for these effects were zoledronate >> pamidronate ≥ alendronate > risedronate. On the other hand, for the ABREs of these NBPs and clodronate (evaluated by their ability to form a BP band in the tibia) the relative potencies were zoledronate >> risedronate = alendronate > pamidronate >> clodronate. Clodronate, when injected together with an NBP, reduced or prevented the inflammation and necrosis induced by the NBP, but had little or no effect on its ABRE. Clodronate reduced the amounts of NBPs retained in the ear tissues. When each agent was intraperitoneally injected, clodronate reduced the amounts of NBPs retained in the liver, spleen, and lung. In the following paragraphs we discuss these results.

BP band formation and ABREs of NBPs and clodronate.

The known relative potencies for the ABREs of BPs are zoledronate >> risedronate > alendronate > pamidronate >> clodronate (Fig. 1). Currently, it is believed that the major pharmacological target of NBPs is the FPP synthase within osteoclasts [3]. However, the ABREs of NBPs could be influenced by (i) their affinities (or binding) to BHA and (ii) their release from the BHA beneath osteoclasts and their subsequent incorporation into the osteoclasts themselves. After intravenous injection into mice, both NBPs and non-NBPs rapidly disappear from the blood (within 2 h), and they bind similarly to bone and are retained there for a long period (> 360 days) [31]. The NBPs bound to bone are released during bone resorption by osteoclasts [35]. Under strong acidic conditions beneath osteoclasts, the released NBPs may be similarly incorporated into osteoclasts via passive diffusion and/or endocytosis [36, 37], because binding of protons to their phosphate residues reduces their polarity (hence making them lipophilic and able to be incorporated into cells). The relative abilities to inhibit FPP synthase *in vitro* are zoledronate >> risedronate > alendronate > pamidronate [38]. Thus, the abilities of NBPs to inhibit FPP synthase are a good reflection of their ABREs. These reported relative abilities are nearly the same as those found in the present study for BP band formation in the tibias of mice. Non-NBPs that had become bound to BHA might be similarly incorporated into osteoclasts via passive diffusion, but they are converted into cytotoxic ATP analogues within osteoclasts [2, 3], indicating that the molecular mechanisms underlying the ABREs of NBPs and non-NBPs are different. In line with the weak ABRE of clodronate, its ability to form BP bands was also weak (Fig. 6). These findings indicate that BP band formation is a good marker for the evaluation of the ABREs of BPs.

Mechanism underlying the inflammatory and necrotic actions of NBPs.

Under neutral conditions, both NBPs and non-NBPs are highly polarized, and they would be expected hardly to enter cells at all across the cell membrane. Indeed non-NBPs rapidly disappear from the blood without retention in non-calcified tissues [31]. In contrast, significant amounts of NBPs (alendronate or pamidronate) have been reported to be retained within some non-calcified tissues in rats and mice (liver, spleen, or lung) either for several hours [32] or for several days [31]. These results suggest that NBPs, but not non-NBPs, are incorporated into cells in non-calcified tissues under neutral conditions. It is known that ionized substances require specific molecules if they are to enter cells (transporters or related proteins). The cholesterol synthesis pathway is widely distributed in eukaryotic cells, and cholesterol is an essential membrane constituent. Thus, inhibition of FPP synthase in this pathway by NBPs may contribute to an impairment of cell functions