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Augmentation of Lipopolysaccharide-Induced Production of IL-1 α and IL-1 β in Mice Given Intravenous Zoledronate (a Nitrogen-Containing Bisphosphonate) and Its Prevention by Clodronate (a Non-nitrogen-containing Bisphosphonate)

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Bisphosphonates (BPs) bind strongly to bone and exhibit long-acting anti-bone-resorptive effects. Among BPs, nitrogen-containing BPs (N-BPs) have far stronger anti-bone-resorptive effects than non-N-BPs. However, N-BPs induce acute inflammatory reactions (fever, arthralgia and myalgia, *etc.*) after their first injection. The mechanisms underlying these side effects remain unclear. Zoledronate (one of the most potent N-BPs) is given intravenously to patients, and the side-effect incidence is reportedly the highest among N-BPs. Our murine experiments have clarified that (a) *intraperitoneally* injected N-BPs induce various inflammatory reactions, including a production of interleukin-1 (IL-1) (a typical inflammatory cytokine), and these inflammatory reactions are weak in IL-1-deficient mice, (b) *subcutaneously* injected N-BPs induce inflammation/necrosis at the injection site, (c) lipopolysaccharide (LPS; a cell-wall component of Gram-negative bacteria) and N-BPs mutually augment their inflammatory/necrotic effects, (d) the non-N-BP clodronate can reduce N-BPs' inflammatory/necrotic effects. However, there are few animal studies on the side effects of *intravenously* injected N-BPs. Here, we found in mice that (i) intravenous zoledronate exhibited weaker inflammatory effects than intraperitoneal zoledronate, (ii) in mice given intravenous zoledronate, LPS-induced production of IL-1 α and IL-1 β was augmented in various tissues, including bone, resulting in them increasing in serum, and (iii) clodronate (given together with zoledronate) prevented such augmentation and enhanced, slightly but significantly, zoledronate's anti-bone-resorptive effect. These results suggest that infection may be a factor promoting the acute inflammatory side effects of N-BPs *via* augmented production of IL-1 in various tissues (including bone), and that clodronate may be useful to reduce or prevent such side effects.

Key words bisphosphonate; zoledronate; clodronate; interleukin-1 (IL-1); lipopolysaccharide (LPS); side effect

INTRODUCTION

Bisphosphonates (BPs), chemicals with a non-hydrolysable P-C-P bond, are analogues of pyrophosphate (PPi), which has a hydrolysable P-O-P bond. Since Fleisch and his coworkers began the research on the effects of bisphosphonates (BPs) on bones,¹⁾ many derivatives have been synthesized by modifying the central carbon (Fig. 1). Interestingly, the BPs that have a nitrogen-containing side-chain (abbreviated as N-BPs) exhibit far stronger anti-bone-resorptive effects than the BPs that lack such a nitrogen-containing side-chain (non-N-BPs).²⁾ Both N-BPs and non-N-BPs bind strongly to bone hydroxyapatite (see Table 2 in Endo *et al.*).³⁾ Thus, they accumulate within bones upon repeated administration.⁴⁾

Now, BPs have become the most widely used drugs for bone diseases in which there is enhanced bone resorption. However, the side effects of N-BPs—such as acute influenza-like inflammation (fever, joint pain, and muscle pain, *etc.*), lesions of digestive organs, and osteonecrosis of the jaw—are a serious concern. Regarding the acute inflammation, it has

been reported that (i) fever occurs in about 30% or more of patients receiving an intravenous N-BP *for the first time* in most cases,^{5,6)} (ii) among children treated with intravenous zoledronic acid, acute flu-like reactions are seen in as many as 85%,⁷⁾ with some severe or fatal cases occurring, albeit rarely,^{8,9)} and (iii) high doses of oral N-BPs also cause flu-like signs although the incidence is low.¹⁰⁾ However, the mechanisms underlying these side effects have still not been established. It is notable that such inflammatory side effects have not been reported in patients given the non-N-BP clodronate.

Our previous murine experiments have clarified that (i) *intraperitoneally* (i.p.) injected N-BPs induce various inflammatory reactions, including the production of interleukin-1 (IL-1) (a typical inflammatory cytokine with a pyrogenic action) in various soft tissues (such as liver, spleen, and lung),^{11–15)} and that such inflammatory reactions are weak in IL-1-deficient mice,^{13,16)} (ii) lipopolysaccharide (LPS, a cell-wall component of Gram-negative bacteria) and N-BPs mutually augment their inflammatory effects,^{13–15,17)} (iii) *locally* injected N-BPs induce inflammation and/or necrosis at the injection sites when given

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Fig. 1. Structures of BPs

BPs, with a non-hydrolysable P-C-P structure, are analogs of pyrophosphoric acid, which has a hydrolysable P-O-P structure. Pamidronate, alendronate, ibandronate, risedronate, zoledronate, and minodronate are N-BPs. Etidronate and clodronate are non-N-BPs. Relative potencies of the anti-bone-resorptive effects of BPs (with etidronate being tentatively indicated as 1.0) are shown within (). The relative potencies of the anti-bone-resorptive effects of BPs were estimated in experiments on rats (Geddes *et al.*, 2005).

at a high concentration,^{18,19} (iv) the non-N-BP clodronate can reduce or prevent the systemic and local inflammatory/necrotic effects of N-BPs^{18–21} by inhibiting the intracellular uptake of N-BPs through phosphate transporters.^{3,21–24}

As described above, the acute inflammatory side effects of N-BPs occur after *intravenous* (i.v.) injection. However, there are few animal studies on the effects of i.v. injected N-BPs. Generally speaking, in animal experiments, many drugs exhibit stronger pharmacological effects after their i.v. injection than after their i.p. injection. Because of the high affinity of BPs for bone hydroxyapatite, it is likely that i.v. injected N-BPs disappear from the circulation more rapidly than i.p. injected N-BPs, possibly resulting in weaker inflammatory effects. Zoledronate, the most potent N-BP, is given i.v. to patients with various bone diseases, and acute inflammatory side effects occur after its initial injection in about 1/2 of the patients.²⁵ Here, we examined the following questions (based on IL-1 being a known mediator of inflammation in mice). (i) Does i.v. injected zoledronate exhibit IL-1-producing effects? (ii) Does LPS modulate such effects of i.v. zoledronate? (iii) Does clodronate modulate such effects of i.v. zoledronate?

MATERIALS AND METHODS

Mice and Reagents ddY mice (male) were obtained from SLC (Hamamatsu, Japan). Zoledronate and clodronate were from Toronto Research Chemicals Inc. (North York, ON, Canada) and Sigma-Aldrich (St. Louis, MO, U.S.A.), respectively. These drugs were dissolved in sterile saline, with the pH of the solutions being adjusted to 7 with NaOH. *Escherichia coli* O55:B5 LPS (prepared by phenol extraction) was purchased from Sigma. Experimental protocols are described in the text or in the legend to the figure relating to each experiment. All

experiments complied with Regulations for Animal Experiments and Related Activities at Tohoku University.

Injection of Reagents into Mice Each reagent (0.1 mL/10 g body weight) was injected either intravenously (*via* a tail vein) (i.v.) or i.p. Experimental protocols and the concentration of each reagent and its rationale are described in the text or in the legend to the Figure relating to each experiment.

Determination of Exudate in Thorax After the thorax had been opened with scissors, the exudate present in the thorax was absorbed using small pre-weighed pieces of filter paper, and the amount of exudate was measured as the increase in the weight of the filter paper.²⁰

Determination of IL-1 α and IL-1 β in Serum Blood was collected directly into test tubes following decapitation. Serum was recovered by centrifugation at 2000 $\times g$ at 4°C, then stored at –80°C until used.¹⁴ The IL-1 α and IL-1 β in the serum were assayed using enzyme-linked immunosorbent assay (ELISA) kits (BioLegend, San Diego, CA, U.S.A.).

Determination of IL-1 α and IL-1 β in Tissues Frozen tissues (liver, lung, and spleen) were homogenized in RPMI 1640 solution containing Triton X-100 (5 μ L/mL), *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES) (10 μ mol/mL), bovine serum albumin (100 μ g/mL), gentamicin sulfate (50 μ g/mL), and proteinase inhibitor cocktail (10 μ L/mL) (Sigma-Aldrich). The protease inhibitor cocktail contains 4-(2-aminoethyl)benzenesulfonyl fluoride, aprotinin, leupeptin, bestatin, pepstatin A, and E-64. The supernatant obtained by centrifugation (10000 $\times g$ for 10 min at 4°C) of the homogenate was then assayed for IL-1 α and IL-1 β using ELISA kits (BioLegend). The antibodies used in these kits recognize both the mature types and pro-types of IL-1 α and IL-1 β . Except for the makers of the kits and the proteinase

inhibitor cocktail, the details of the procedures used here are essentially the same as those described previously.^{14,15)}

Anti-bone-resorptive Effects of BPs 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.^{26–28)} Hence, we can estimate the anti-bone-resorptive effects of N-BPs by using the BP band as a marker. Briefly, each BP solution was i.v. injected (0.2 mL/mouse) into young male mice (5 weeks old). The mice were decapitated three 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 s.²⁶⁾ 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 from that obtained from corresponding areas of normal tibias [$n = 3$ mice (*i.e.*, 6 tibias)].

Data Analysis Experimental values are given as the mean $\pm$ standard deviation (S.D.). The statistical significance of the difference between two means was evaluated using a Bonferroni multiple-comparison test after ANOVA. Data were analyzed using Instat software (GraphPad Software Inc., La Jolla, CA, U.S.A.).

RESULTS

Inflammatory or Toxic Effects of i.v. and i.p. Injected Zoledronate I.p. injection is a widely used method for investigating the systemic effects of a test reagent. Our group has reported that splenomegaly and an accumulation of the exudate in thorax are commonly seen as inflammatory reactions when N-BPs (alendronate, ibandronate, incadronate, and minodronate) are i.p. injected into mice.^{13,29)} Thus, to examine how i.v. injection of zoledronate might produce systemic inflammatory effects, we first compared the reactions to i.p. and i.v. injections of zoledronate. As shown in Fig. 2, i.p. injected 2 mM zoledronate exhibited inflammatory (splenomegaly) or toxic (accumulation of exudate in thorax) effects. Notably, however, i.v. injected 2 mM zoledronate did not induce such effects, although i.v. injected zoledronate did induce splenomegaly at 4 mM. These results indicate that the inflammatory and/or toxic effects of i.v. injected zoledronate are weaker than those of i.p. injected zoledronate.

Effects of i.v. and i.p. Injected Zoledronate on IL-1 α and IL-1 β in Tissues and Serum We previously reported that i.p. injected alendronate (a typical N-BP used for osteoporosis) increases IL-1 β in various soft tissues, such as liver, spleen, and lung, but not in serum.^{14,15)} In the present study, we compared the effects of i.v. and i.p. injected zoledronate on IL-1 α and IL-1 β in serum and in various soft tissues (spleen, liver, lung, and skeletal muscle) and in a hard tissue, bone. As shown in Figs. 3A and 3B, when injected i.p. (2 mM) or i.v. (2 and 4 mM), zoledronate produced no notable effects on the levels of IL-1 α and IL-1 β in serum and the tissues tested. These results indicate that the effects of i.v. injected zoledronate, even if given at 4 mM, on the production of IL-1 α and

Fig. 2. Inflammatory Effects of i.v. and i.p. Injected Zoledronate (Zol)

Zol (2 or 4 mM) was i.v. or i.p. injected, and 3 d later, the indicated values were measured. * $p < 0.05$ vs. Saline, $n = 4$.

IL-1 β are very weak, if any.

Effects of i.v. LPS on IL-1 α and IL-1 β Productions in Mice Pretreated with i.v. Zoledronate In our previous studies, in which both alendronate (4 mM) and LPS (10 μ g/mL) were i.p. injected, alendronate by itself did not increase the serum levels of IL-1 α and IL-1 β , but the LPS-induced elevations of serum IL-1 α and IL-1 β were markedly augmented in mice pretreated with alendronate 3 d before the LPS injection.^{12,13,15)}

In the present study, we examined the effects of i.v. injected zoledronate and LPS. LPS (10 μ g/mL) was i.v. injected into control mice or mice treated with an i.v. injection of 4 mM zoledronate 1, 2 or 3 d before the LPS injection. As shown in Figs. 4A and 4B, LPS alone, 2 h after its injection, increased one or both of IL-1 α and IL-1 β in serum and in all of the tissues tested. With the exception of the liver, the LPS-induced increases in IL-1 α and/or IL-1 β were augmented in the serum and all of the tissues tested in mice pretreated with zoledronate 2 or 3 d before the LPS injection. Such augmentation was particularly marked in the tibia and spleen. The results obtained in the liver were essentially the reverse of those in the other tissues. At present, we have no data to explain this difference (see Discussion). In any event, these results suggest that (i) the LPS-induced productions of IL-1 α and IL-1 β are augmented in various (possibly many) tissues in zoledronate-treated mice, and (ii) this augmentation may be sufficient to lead to an increase in IL-1 α and IL-1 β in the serum.

Effects of Clodronate on the Augmented Production of IL-1 α and IL-1 β Induced by Zoledronate Plus LPS We previously reported that the inflammatory effects of the i.p. injected N-BPs tested (4 mM) are reduced by co-injection with clodronate (1–16 mM),²⁰⁾ and that the inflammatory and/or necrotic effects of locally injected N-BPs, including zoledronate, are likewise reduced by co-injection with clodronate.^{18,19,21,22)} So, we compared the effects of clodronate on the augmented production of IL-1 α and IL-1 β seen in mice given i.v. zoledronate and then (3 d later) i.v. LPS. In this experiment, we measured the levels of IL-1 α and IL-1 β in serum, tibia, and spleen. As shown in Fig. 5, 20 mM clodronate had no significant effects on the LPS-induced production of IL-1 α and IL-1 β . However, clodronate markedly reduced the augmented production of IL-1 α and/or IL-1 β in the tibia and spleen induced by zoledronate followed by LPS, and it also reduced the levels of

Fig. 3. Effects of i.v. and i.p. Injected Zol on IL-1 α and IL-1 β

Zol (2 or 4 mM) was i.v. or i.p. injected, and 3 d later, blood and tissues were sampled for measuring IL-1 α and IL-1 β . A: data from serum, tibia, and spleen. B: data from liver, lung, and pectoralis. * $p < 0.05$, $n = 4$. There were no significant differences between control saline groups and other groups.

both IL-1 α and IL-1 β in the serum to the levels induced by LPS alone.

Effect of Clodronate on the Anti-bone-resorptive Effect of i.v. Injected Zoledronate As described above, i.v. injected clodronate reduced the inflammatory effects of i.v. injected zoledronate. Thus, it was important to examine whether i.v. injected clodronate might modulate the anti-bone-resorptive effect of i.v. injected zoledronate. The anti-bone-resorptive effects of BPs are easily estimated by quantifying the BP-band (see Methods). Using this method, we previously showed that i.p. injection of a mixture containing 0.1 mM zoledronate and 10 mM clodronate (the ratio of zoledronate to clodronate being 1:100) did not reduce the anti-bone-resorptive effect of zoledronate (Oizumi *et al.*).¹⁸⁾ In the present study, we examined the effect of i.v. injected 10 mM clodronate on the BP-band produced by i.v. injected 1 mM zoledronate (the ratio of zoledronate to clodronate being 1:10). As shown in Fig. 6: (i) 1 mM zoledronate produced a clear BP-band in the tibia,

while (ii) no clear BP-band was detected in the tibias of mice given clodronate, probably because the anti-bone resorptive activity of clodronate is about 1/3000 that of zoledronate (Fig. 1), and (iii) to the naked eye, there was no clear difference in BP-bands between the two groups, zoledronate vs. zoledronate + clodronate, but the mean gray value analysis (see Materials and Methods) indicated that the anti-bone-resorptive effect of zoledronate was not reduced (indeed, it was slightly, but significantly, enhanced) by co-administration with clodronate.

DISCUSSION

Summary of the Present Findings In the present study in mice, we found (i) surprisingly, i.v. injected zoledronate exhibited weaker inflammatory effects than i.p. injected zoledronate, (ii) in mice given i.v. zoledronate, the LPS-induced increase in the production of IL-1 α and IL-1 β in various

Fig. 4. Effects of LPS on IL-1 α and IL-1 β in Mice Pretreated with i.v. Zol

Zol (4 mM) was i.v. injected into mice. At 24, 48, or 72 h after the zoledronate injection, LPS (10 µg/mL) was i.v. injected into the mice, and 2 h later blood and tissues were sampled for measuring IL-1 α and IL-1 β . A: data from serum, tibia, and spleen. B: data from liver, lung, and pectoralis. * p < 0.05, ** p < 0.01, *** p < 0.001, NS: not significant, n = 4.

tissues, including bone, was augmented, resulting in them increasing in the serum, (iii) clodronate (given together with zoledronate) prevented such augmentation of IL-1 α and IL-1 β productions, and (iv) unexpectedly and interestingly, the anti-bone-resorptive effect of i.v. injected zoledronate was enhanced, albeit slightly, by i.v. injected clodronate. In the following paragraphs, we discuss these findings.

Effects of i.v. Injection of Zoledronate In animal experiments, many drugs exhibit stronger effects when they are i.v. injected than when they are i.p. injected. Thus, it was surprising that the inflammatory effects of zoledronate were weaker when it was i.v. injected than those when it was i.p. injected.

This “unusual” result may be related to the potent affinity of zoledronate for bone hydroxyapatite, *i.e.*, i.v. injected zoledronate may bind rapidly to bone and quickly disappear from the circulation, resulting in a rapid reduction in its intracellular uptake and thus to reduced inflammatory effects. This unique property of i.v. zoledronate (and perhaps other N-BPs, too) would seem likely to contribute to its safety in clinical use when zoledronate is administered to patients by slow intravenous drip. Nevertheless, the incidence of acute inflammatory side effects of zoledronate is very high. Thus, it is very important to clarify the mechanism underlying the acute inflammatory side effects of zoledronate as well as of other N-BPs.

Fig. 5. Effects of Clodronate (Clo) on the Augmented Production of IL-1 α and IL-1 β Induced by Zol Plus LPS

Clo alone (20mM), Zol alone (4mM), or a mixture of the two (containing those concentrations) was i.v. injected. At 72h after the injection, LPS (10µg/mL) was i.v. injected into the mice, and 2h later blood and tissues were sampled for measuring IL-1α and IL-1β. A: data from serum, tibia, and spleen. B: data from liver, lung, and pectoralis. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, NS: not significant, $n = 4$.

Fig. 6. Effect of Clodronate (Clo) on Anti-bone Resorptive Effect of i.v. Injected Zol

Zol (1 mM), Clo (10 mM), or a mixture containing 1 mM Zol and 10 mM Clo was i.v. injected (0.2 mL/mouse) into young male mice (5 weeks old). Three weeks later, tibias were removed and subjected to the estimation of anti-bone resorptive effects using the BP-band as a marker (see Methods). *** $p < 0.001$, NS: not significant, $n = 3$ mice (i.e., 6 tibias).

Effects of LPS on the Production of IL-1 α and IL-1 β in Mice Pretreated with i.v. Zoledronate The inflammatory effects of i.v. zoledronate by itself were very weak in the present study. Thus, it might be anticipated that an unknown factor(s) can, under some circumstances, promote or augment the weak inflammatory action of i.v. zoledronate. We found in our previous studies that the inflammatory effects of i.p. injected N-BPs were augmented by LPS or IL-1,^{13–15,17)} and that i.p. injected alendronate (one of the widely used N-BPs) somewhat increased IL-1 β levels (by two times at most) in soft tissues (such as liver, spleen, and lung) *via* formation of IL-1 β .

mRNA.^{14,15}) In the present study, we could not detect such an increase in IL-1 α or IL-1 β protein in mice treated with i.v. or i.p. zoledronate alone (Figs. 3A, B). However, we established that their LPS-stimulated production was markedly augmented in mice previously (24 or 72 h) treated with i.v. zoledronate (Figs. 4A, B). Notably, in addition to various soft tissues, the LPS-induced productions of IL-1 α and IL-1 β were markedly augmented in a bone (the tibia, to which zoledronate binds strongly) (Fig. 4A). These results suggest that an increased production of IL-1 α and/or IL-1 β secondary to LPS or infection in various tissues, including bones, might be causally

involved in the acute inflammatory side effects that can occur in patients treated with zoledronate or other N-BPs.

Mechanism Underlying the Acute Inflammatory Side Effects of i.v. Zoledronate IL-1 α and IL-1 β are typical inflammatory cytokines with pyrogenic activity,³⁰⁾ and they share common receptors in producing their effects. Interestingly, the augmented production of IL-1 α and IL-1 β in zoledronate-pretreated mice was particularly marked in bone (tibia) and spleen, both of which are hematopoietic organs. If we express the amounts of these cytokines in the tibia in terms of “weight of bone marrow,” the values may become about 10 times or more larger. In addition, bone is the largest tissue in the body. Thus, bone marrow might be an important source of IL-1 α and IL-1 β . Interestingly, bone-bound bisphosphonates have been shown to inhibit the growth of adjacent non-bone cells *in vitro*,³¹⁾ suggesting that bone-bound N-BPs can be released, although the underlying mechanism is not clear. Thus, it is possible that bone-bound zoledronate can stimulate adjacent cells to increase their production of IL-1 α and IL-1 β . We are now doing experiments to clarify the mechanism underlying the augmented production of IL-1 α and IL-1 β in mice pretreated with zoledronate, and the results so far suggest that the expression of TLR4, the receptor for LPS, is upregulated by N-BPs (manuscript in preparation).

Effect of Clodronate on the Increased IL-1 α and IL-1 β Production Induced by Zoledronate Plus LPS Our pharmacological results suggest that N-BPs may enter soft-tissue cells *via* the phosphate transporter families SLC20 and/or SLC34, and that clodronate inhibits this entry and thereby reduces or prevents the inflammatory and/or necrotic effects of N-BPs.^{21,22)} SLC20 transporters are known to be present ubiquitously throughout the body.^{32,33)} In the present study, we found that 20mM clodronate, when co-injected with 4mM zoledronate, can reduce the augmented production of IL-1 α and IL-1 β in bone and spleen induced when i.v. injected zoledronate is followed by LPS, resulting in their marked reduction in the serum (Fig. 5). Thus, it might be expected that N-BP-induced acute inflammatory side effects could be prevented or reduced by co-administration of an N-BP and clodronate.

Interestingly, some animal and human studies suggest that non-N-BPs, including clodronate, have analgesic effects that are independent of their anti-bone-resorptive effect.^{34,35)} Neuronal vesicles accumulate the pain transmitters glutamic acid and ATP through transporters belonging to the SLC17 family.^{36,37)} Recently, we demonstrated that clodronate may enter neurons through SLC20/34, then inhibit SLC17-mediated transport of glutamate and/or ATP, resulting in their decrease, and thereby produce analgesic effects.^{19,24)} Thus, clodronate may also be effective at preventing or reducing the pain in muscles and joints induced by N-BPs.

Incidentally, although *in vitro* studies suggest that statins might be effective at reducing or preventing N-BP-induced acute inflammation *via* inhibition of $\gamma\delta$ T-cells,^{38,39)} such a utility is not observed in clinical use in children.^{40,41)} Dexamethasone (a steroid) is not effective, either,⁴²⁾ whereas acetaminophen (acting mainly on the central nervous system) and ibuprofen (a non-steroidal anti-inflammatory agent) have been reported to be effective.^{41,43)}

Effects of Clodronate on the Anti-bone-resorptive Effects of N-BPs The affinity of clodronate for bone is very

low, possibly the lowest, among the clinically employed BPs (see Table 2 in Endo *et al.*).³⁾ Thus, when an N-BP is given together with clodronate, clodronate does not competitively inhibit the binding of the N-BP to bones, and the anti-bone-resorptive effect of the N-BP is not profoundly affected.³⁾ Indeed, in contrast to its ability to induce inflammatory side effects, i.p. injected clodronate does not reduce the anti-bone resorptive effects of N-BPs, such as zoledronate, alendronate, and risedronate.^{18,26)} Unexpectedly and very interestingly, in the present study, the anti-bone resorptive activity of i.v. injected zoledronate was slightly but significantly enhanced by co-injection of clodronate (Fig. 6). We can speculate that this enhancement might be due to a higher blood level of zoledronate resulting from an inhibition of its uptake by soft-tissue cells *via* a clodronate-induced inhibition of the phosphate transporters SLC20 and/or SLC34, as described above. In addition, because the affinity of clodronate for bone hydroxyapatite is far weaker than that of zoledronate (see Table 2 in Endo *et al.*),³⁾ competitive inhibition of zoledronate's binding to bone by clodronate may be very weak if any. Thus, clodronate, when combined with an N-BP, can be expected to not only reduce or prevent inflammatory side effects of the N-BP but also to enhance the potent anti-bone-resorptive effect of the N-BP.

Effects of Zoledronate on Liver In liver, unlike in other tissues, the LPS-induced increase in IL-1 α and IL-1 β was not augmented in mice pretreated with zoledronate (indeed, it was reduced, or tended to be reduced) (Fig. 4B). This result is puzzling: zoledronate appears to limit or impair the ability of LPS to increase IL-1 production specifically in the liver (among the tissues tested so far). At present, we have no relevant data to guide us towards the underlying mechanism. However, we can say the following. Although gastrointestinal lesions are the major side effects of N-BPs on soft tissues, Goossens *et al.*⁴⁴⁾ pointed out that hepatitis, although infrequent, is also induced by N-BPs (including zoledronate as well as alendronate, risedronate, and ibandronate). The liver is the drug-metabolizing organ, and systemically administered N-BPs gather in this organ. Thus, the liver might also be an important target in the direct toxic effect of N-BPs. However, we will need detailed studies to explore whether this has any relevance to the above puzzling result.

CONCLUSION

The results in the present study suggest that infection may be a factor promoting the acute inflammatory side effects of N-BPs *via* augmented production of IL-1 in various tissues, including bone, and that clodronate administration might be a useful way of preventing or reducing the side effects of N-BPs while preserving or enhancing their strong anti-bone-resorptive effects.

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Conflict of Interest The authors declare no conflict of interest.

REFERENCES

- 1) Fleisch H. Diphosphonates: history and mechanisms of actions. *Metab. Bone Dis. Relat. Res.*, **3**, 279–287 (1981).
- 2) Rogers MJ, Crockett JC, Coxon FP, Mönkkönen J. Biochemical and molecular mechanisms of action of bisphosphonates. *Bone*, **49**, 34–41 (2011).
- 3) Endo Y, Kumamoto H, Nakamura M, Sugawara S, Takano-Yamamoto T, Sasaki K, Takahashi T. Underlying mechanisms and therapeutic strategies for bisphosphonate-related osteonecrosis of the jaw (BRONJ). *Biol. Pharm. Bull.*, **40**, 739–750 (2017).
- 4) Geddes AD, D'Souza SM, Ebetino FH, Kenneth JJ. Bisphosphonates: structure–activity relationships and therapeutic implications. *Bone and Mineral Research*. (Heershe NM, Kanis JK ed). Vol. 8, Elsevier Science BV, Amsterdam, Holland, pp. 265–306 (1994).
- 5) Adami S, Bhalla AK, Dorizzi R, Montesanti F, Rosini S, Salvagno G, Lo Cascio V. The acute phase response after bisphosphonate administration. *Calcif. Tissue Int.*, **41**, 326–331 (1987).
- 6) Adami S, Zamberlan N. Adverse effects of bisphosphonate: a comparative review. *Drug Saf.*, **14**, 158–170 (1996).
- 7) Höglér W, Yap F, Little D, Ambler G, McQuade M, Cowell CT. Short-term safety assessment in the use of intravenous zoledronic acid in children. *J. Pediatr.*, **145**, 701–704 (2004).
- 8) Trivedi S, Al-Nofal A, Kumar S, Tripathi S, Kahoud RJ, Tebben PJ. Severe non-infective systemic inflammatory response syndrome, shock, and end-organ dysfunction after zoledronic acid administration in a child. *Osteoporos. Int.*, **27**, 2379–2382 (2016).
- 9) Munns CF, Rauch F, Mier RJ, Glorieux FH. Respiratory distress with pamidronate treatment in infants with severe osteogenesis imperfecta. *Bone*, **35**, 231–234 (2004).
- 10) Reginster JY, Adami S, Lakatos P, Greenwald M, Stepan JJ, Silverman SL, Christiansen C, Rowell L, Mairon N, Bonvoisin B, Drezner MK, Emkey R, Felsenberg D, Cooper C, Delmas PD, Miller PD. Efficacy and tolerability of once-monthly oral ibandronate in postmenopausal osteoporosis: 2 year results from MOBILE study. *Ann. Rheum. Dis.*, **65**, 654–661 (2006).
- 11) Endo Y, Nakamura M, Kikuchi T, Shinoda H, Takeda Y, Nitta Y, Kumagai K. Aminoalkylbisphosphonates, potent inhibitors of bone resorption, induce a prolonged stimulation of histamine synthesis and increase macrophages, granulocytes and osteoclasts *in vivo*. *Calcif. Tissue Int.*, **52**, 248–254 (1993).
- 12) Sugawara S, Shibazaki M, Takada H, Kosugi H, Endo Y. Contrasting effects of an aminobisphosphonate, a potent inhibitor of bone resorption, on lipopolysaccharide-induced production of interleukin-1 and tumour necrosis factor α in mice. *Br. J. Pharmacol.*, **125**, 735–740 (1998).
- 13) Yamaguchi K, Motegi K, Iwakura Y, Endo Y. Involvement of interleukin-1 in the inflammatory actions of aminobisphosphonates in mice. *Br. J. Pharmacol.*, **130**, 1646–1654 (2000).
- 14) Deng X, Yu Z, Funayama H, Shoji N, Sasano T, Iwakura Y, Sugawara S, Endo Y. 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.*, **213**, 64–73 (2006).
- 15) Shikama Y, Nagai Y, Okada S, Oizumi T, Shimauchi H, Sugawara S, Endo Y. Pro-IL-1 β accumulation in macrophages by alendronate and its prevention by clodronate. *Toxicol. Lett.*, **199**, 123–128 (2010).
- 16) Deng X, Yu Z, Funayama H, Yamaguchi K, Sasano T, Sugawara S, Endo Y. Histidine decarboxylase-stimulating and inflammatory effects of alendronate in mice: involvement of mevalonate pathway, TNF α , macrophages, and T-cells. *Int. Immunopharmacol.*, **7**, 152–161 (2007).
- 17) Funayama H, Mayanagi H, Takada H, Endo Y. Elevation of histidine decarboxylase activity in the mandible of mice by *Prevotella intermedia* lipopolysaccharide and its augmentation by an aminobisphosphonate. *Arch. Oral Biol.*, **45**, 787–795 (2000).
- 18) Oizumi T, Yamaguchi K, Funayama H, Kuroishi T, Kawamura H, Sugawara S, Endo Y. 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. *Basic Clin. Pharmacol. Toxicol.*, **104**, 384–392 (2009).
- 19) Shima K, Tsuchiya M, Oizumi T, Takano-Yamamoto T, Sugawara S, Endo Y. Inflammatory effects of nitrogen-containing bisphosphonates (N-BPs): modulation by non-N-BPs. *Biol. Pharm. Bull.*, **40**, 25–33 (2017).
- 20) Endo Y, Shibazaki M, Yamaguchi K, Nakamura M, Kosugi H. Inhibition of inflammatory actions of aminobisphosphonates by dichloromethylene bisphosphonate, a non-aminobisphosphonate. *Br. J. Pharmacol.*, **126**, 903–910 (1999).
- 21) Kiyama T, Tsuchiya M, Okada S, Oizumi T, Yamaguchi K, Sasaki K, Sugawara S, Endo Y. Phosphonocarboxylates can protect mice against the inflammatory and necrotic side effects of nitrogen-containing bisphosphonates by inhibiting their entry into cells *via* phosphate transporters. *Biol. Pharm. Bull.*, **39**, 712–720 (2016).
- 22) Okada S, Kiyama T, Sato E, Tanaka Y, Oizumi T, Kuroishi T, Takahashi T, Sasaki K, Sugawara S, Endo Y. Inhibition of phosphate transporters ameliorate the inflammatory and necrotic side effects of the nitrogen-containing bisphosphonate zoledronate in mice. *Tohoku J. Exp. Med.*, **231**, 145–158 (2013).
- 23) Shima K, Nemoto W, Tsuchiya M, Tan-No K, Takano-Yamamoto T, Sugawara S, Endo Y. The bisphosphonates clodronate and etidronate exert analgesic effects by acting on glutamate- and/or ATP-related pain transmission pathways. *Biol. Pharm. Bull.*, **39**, 770–777 (2016).
- 24) Kato Y, Hiasa M, Ichikawa R, Hasuzawa N, Kadowaki A, Iwatsuki K, Shima K, Endo Y, Kitahara Y, Inoue T, Nomura M, Omote H, Moriyama Y, Miyaji T. Identification of a vesicular ATP release inhibitor for the treatment of neuropathic and inflammatory pain. *Proc. Natl. Acad. Sci. U.S.A.*, **114**, E6297–E6305 (2017).
- 25) Popp AW, Senn R, Curkovic I, Senn C, Buffat H, Popp PF, Lipuner K. Factors associated with acute-phase response of bisphosphonate-naïve or pretreated women with osteoporosis receiving an intravenous first dose of zoledronate or ibandronate. *Osteoporos. Int.*, **28**, 1995–2002 (2017).
- 26) Monma Y, Funayama H, Mayanagi H, Endo Y. Effects of weekly administrations of alendronate + clodronate on young mouse tibia: localized action at the proximal growth plate. *Calcif. Tissue Int.*, **74**, 115–121 (2004).
- 27) Funayama H, Ohsako M, Monma Y, Mayanagi H, Sugawara S, Endo Y. Inhibition of inflammatory and bone-resorption-inhibitory effects of alendronate by etidronate. *Calcif. Tissue Int.*, **76**, 448–457 (2005).
- 28) Yu Z, Funayama H, Deng X, Kuroishi T, Sasano T, Sugawara S, Endo Y. Comparative appraisal of clodronate, aspirin and dexamethasone as agents reducing alendronate-induced inflammation in a murine model. *Basic Clin. Pharmacol. Toxicol.*, **97**, 222–229 (2005).
- 29) Kiyama T, Okada S, Tanaka Y, Kim S, Bando K, Hasegawa M, Yamaguchi K, Takano-Yamamoto T, Sasaki K, Sugawara S, Endo Y. Inflammatory and necrotic effects of minodronate, a nitrogen-containing bisphosphonates, in mice. *Tohoku J. Exp. Med.*, **230**, 141–149 (2013).
- 30) Dinarello CA. A clinical perspective of IL-1 β as the gatekeeper of inflammation. *Eur. J. Immunol.*, **41**, 1203–1217 (2011).
- 31) Cornish J, Bava U, Callon KE, Bai J, Naot D, Reid IR. Bone-bound bisphosphonate inhibits growth of adjacent non-bone cells. *Bone*, **49**, 710–716 (2011).
- 32) Virkki LV, Biber J, Murer H, Forster IC. Phosphate transporters: a tale of two solute carrier families. *Am. J. Physiol. Renal Physiol.*, **293**, F643–F654 (2007).

- 33) Miyamoto K, Haito-Sugino S, Kuwahara S, Ohi A, Nomura K, Ito M, Kuwahata M, Kido S, Tatsumi S, Kaneko I, Segawa H. Sodium-dependent phosphate cotransporters: lessons from gene knockout and mutation studies. *J. Pharm. Sci.*, **100**, 3719–3730 (2011).
- 34) Fujita T, Ohue M, Fujii Y, Miyauchi A, Takagi Y. Comparison of the analgesic effects of bisphosphonates: etidronate, alendronate and risedronate by electroalgotomy utilizing the fall of skin impedance. *J. Bone Miner. Metab.*, **27**, 234–239 (2009).
- 35) Kim S, Seiryu M, Okada S, Kuroishi T, Takano-Yamamoto T, Sugawara S, Endo Y. Analgesic effects of the non-nitrogen-containing bisphosphonates etidronate and clodronate, independent of anti-resorptive effects on bone. *Eur. J. Pharmacol.*, **699**, 14–22 (2013).
- 36) Debrek L, Thor P. Glutamate NMDA receptors in pathology and pharmacotherapy of selected nervous system diseases. *Postepy Hig. Med. Dosw.*, **65**, 338–346 (2011).
- 37) Omote H, Moriyama Y. Vesicular neurotransmitter transporters: an approach for studying transporters with purified proteins. *Physiology*, **28**, 39–50 (2013).
- 38) Thompson K, Rogers MJ. Statins prevent bisphosphonate-induced $\gamma\delta$ T-cell proliferation and activation *in vitro*. *J. Bone Miner. Res.*, **19**, 278–288 (2004).
- 39) Hewitt RE, Lissina A, Green AE, Slay ES, Price DA, Sewell AK. The bisphosphonate acute phase response: Rapid and copious production of proinflammatory cytokines by peripheral blood $\gamma\delta$ T cells in response to aminobisphosphonates is inhibited by statins. *Clin. Exp. Immunol.*, **139**, 101–111 (2005).
- 40) Srivastava T, Haney CJ, Alon US. Atorvastatin may have no effect on acute phase reaction in children after intravenous bisphosphonate infusion. *J. Bone Miner. Res.*, **24**, 334–337 (2009).
- 41) Silverman SL, Kriegman A, Goncalves J, Kianifard F, Carlson T, Leary E. Effect of acetaminophen and fluvastatin on post-dose symptoms following infusion of zoledronic acid. *Osteoporos. Int.*, **22**, 2337–2345 (2011).
- 42) Billington EO, Horne A, Gamble GD, Maslowski K, House M, Reid IR. Effect of single-dose dexamethasone on acute phase response following zoledronic acid: a randomized controlled trial. *Osteoporos. Int.*, **28**, 1867–1874 (2017).
- 43) Robinson RE, Nahata MC, Hayes JR, Batisky DL, Bates CM, Mahan JD. Effectiveness of pretreatment in decreasing adverse events associated with pamidronate in children and adolescents. *Pharmacotherapy*, **24**, 195–197 (2004).
- 44) Goossens N, Spahr L, Rubbia-Brandt L. Severe immune-mediated drug-induced liver injury linked to ibandronate: a case report. *J. Hepatol.*, **59**, 1139–1142 (2013).