Effect of minodronate, a nitrogen-containing bisphosphonate, on collagen-induced arthritis in mice

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Abstract
Nitrogen-containing bisphosphonates (NBPs) are used as therapeutic agents in many bone disorders. Recent studies have reported side effects associated with NBP, such as exacerbation of inflammation and osteonecrosis. We previously found that the NBP alendronate (ALN) exacerbated inflammation and bone destruction in a mouse model of collagen-induced arthritis (CIA). A newly developed NBP, minodronate (MIN), was found to inhibit osteoclastic bone resorption more strongly than ALN. However, few studies have examined the effects of MIN in CIA mice. To address this issue, in the present study mice were intraperitoneally injected with MIN (4 μmol/kg) once a week starting 7 days before the first sensitization with type II collagen. The MIN-treated group showed higher clinical arthritic scores at every time point and a significant increase in serum concentration of tumor necrosis factor (TNF)-α, while a histological analysis revealed an increase in the amount of trabecular bone growth as compared to the untreated group, indicating inhibition of bone resorption by osteoclasts. Furthermore, MIN enhanced the massive infiltration of neutrophils, CD4-positive cells and T cell receptor (TCR) γδ-positive T cells into the synovium and joint cavity as well as destruction of articular cartilage. The mRNA expression of pro-inflammatory cytokines including interleukin (IL)-17 was upregulated in the synovium of MIN-treated mice, as detected by real time reverse transcription-PCR. These results suggest that MIN exacerbates inflammatory bone diseases by continuous induction of inflammation via pro-inflammatory cytokines such as TNF-α and IL-17.

Keywords: Bisphosphonate, Collagen-induced arthritis, Cytokine, T cell, Osteoclast

Abbreviations:
ALN, alendronate; APC, allophycocyanin; BP, bisphosphonates; CIA, collagen-induced arthritis; Ct, cycle threshold; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; FPP, farnesyl pyrophosphate; Gr-1, gamma response 1; IL, interleukin; FACS, fluorescence-activated cell sorting; micro-CT, micro-computed tomography; MIN, minodronate; MMP, matrix metalloproteinase; NBP, nitrogen-containing bisphosphonates; NK, natural killer; PBS, phosphate-buffered saline; PE, phycoerythrin; qPCR, quantitative real-time PCR; RA, rheumatoid arthritis; TCR, T cell receptor; Th17, helper T17; TNF-α, tumor necrosis factor-α.

Introduction
Rheumatoid arthritis (RA) is characterized by chronic inflammation of the synovium, progressive pannus formation, and ultimate destruction of bone and cartilage [1, 2]. Although the etiology of RA remains unclear, its pathogenesis has been studied extensively in patients as well as in animal models [3-5]. Pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, and IL-6 play critical roles in RA [2, 6, 7]; their production in the synovium is increased in RA patients as well as in animal models of arthritis [4, 8, 9]. IL-1β promotes inflammation and destruction of the synovium, bone, cartilage, and joints in RA patients [10-12], and mediates synovial inflammation and pannus formation [13]; it also induces the expression of other pro-inflammatory cytokines such as TNF-α and IL-6 [14-16].

IL-17-producing helper T (Th)17 cells have recently been found to play a critical role in the development of autoimmune arthritis, including collagen-induced arthritis (CIA) [17-19]. Blocking IL-17 after disease onset prevents cartilage and bone destruction, leading to improvement of clinical symptoms [20, 21]. IL-17 receptor signaling is a critical pathway in the progression of acute synovitis to chronic destructive arthritis [22]. In RA patients, IL-17 is spontaneously produced by the rheumatoid synovium [23] as well as by a variety of cell types, including γδ T cells, natural killer (NK)T cells, NK cells, neutrophils, and eosinophils [24-27]. IL-17 also participates in neutrophil recruitment via chemokine release and stimulation of granulopoiesis [28, 29].

Bisphosphonates (BPs) strongly inhibit bone resorption by osteoclasts [30]. BPs can be classified into nitrogen-containing bisphosphonates (NBPs) and non-nitrogen containing bisphosphonates (non-NBPs) depending on the presence or absence of nitrogen in their side chains. NBPs have more potent anti-bone resorptive effects than non-NBPs [31], but have inflammatory side effects such as fever, jaw osteomyelitis, osteonecrosis, and extramedullary erythropoiesis [32-34]. Our previous study showed that the NBP alendronate (ALN) exacerbated inflammation and bone destruction of the synovium, bone, cartilage, and joints in RA mice. Cellular Immunology & Immunotherapeutics 1: 1-7.

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destruction in a mouse model of CIA [35, 36]. Since April 2009, the NBP minodronate (MIN) has been used for osteoporosis treatment in Japan. Although MIN is at least 10 times more potent than ALN in inhibiting bone resorption in vivo and in vitro [37, 38], MIN-related side effects have not been investigated [39, 40]. We addressed this in the present study by examining how MIN is related to the onset, exacerbation, and persistence of arthritis in a mouse model.

Materials and Methods

The experimental protocols used in this study were reviewed and approved by the Animal Care Committee of Showa University.

Mice and reagents

Male DBA/1 mice (7 weeks old) were obtained from Sankyo Laboratories (Tokyo, Japan), and were maintained under specific pathogen-free conditions. A total of 60 mice were divided into four groups: untreated DBA/1 mice (normal), MIN-treated DBA/1 mice (MIN), untreated CIA mice (CIA w/o MIN), and MIN-treated CIA mice (CIA w/MIN).

Bovine type II collagen solution and complete Freund's adjuvant were purchased from Chondrex (Redmond, WA, USA). MIN was synthesized by Chengdu D-Innovation Pharmaceutical Co. (Chengdu, China) and dissolved in sterile saline, with the pH adjusted to 7 using NaOH.

Monoclonal anti-mouse Ly-6G and Ly-6C (Gr-1), phycoerythrin (PE)-conjugated anti-mouse CD11b, fluorescein isothiocyanate (FITC)-conjugated anti-mouse Gr-1, anti-mouse CD4, and anti-mouse TCR y6 antibodies were purchased from BD Pharmingen (San Diego, CA, USA). Monoclonal anti-mouse allophycocyanin (APC)-conjugated anti-CD3 antibody and anti-rat IgG (isotype control) were purchased from eBioscience (San Diego, CA, USA). Biotinylated goat anti-rat and anti-hamster IgG and avidin–biotin complex kit (ABC Elite) were purchased from Vector Laboratories (Burlingame, CA, USA).

Induction of CIA

Arthritis was induced in DBA/1 mice by injecting a mixture of type II collagen and complete Freund's adjuvant. The emulsion consisting of 1 ml of 0.2% type II collagen in 0.01 M acetic acid and 1 ml of the adjuvant was prepared by homogenization with a sonicator; 50 ml of the mixture was intradermally injected at the base of the tail of 7-week-old DBA/1 mice (first sensitization). Three weeks later, mice were given another injection (second sensitization).

The severity of joint inflammation in each mouse was quantified by scoring each paw on a scale of 0 to 3, as follows: 0 = normal; 1 = erythema and swelling of one digit; 2 = erythema and swelling of two digits; 3 = erythema and swelling of the whole ankle. Incidence and total number of arthritis cases were recorded. Mice were sacrificed for analysis at 2, 4, and 8 weeks after the second sensitization.

BP administration

MIN (4 µmol/kg) was intraperitoneally injected once a week starting 1 week before the first sensitization to eliminate the possibility of bone resorption by osteoclasts. In the CIA w/o MIN group, mice were injected with an equivalent volume of saline.

Cytokine detection by enzyme-linked immunosorbent assay (ELISA)

To detect serum levels of TNF-α and matrix metalloproteinase (MMP)3, mice were bled by cardiac puncture; the blood was allowed to clot at room temperature, and the serum was separated by centrifugation. TNF-α and MMP3 concentrations were measured using mouse ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocols.

Fluorescence-activated cell sorting (FACS)

Synovial fluid was collected from knee joints using a pipette. Cells were separated from the fluid by centrifugation and 2 × 10^6 cells were incubated for 1 h with PE-conjugated anti-CD11b (1:500), FITC-conjugated anti-Gr-1 (1:500), and APC-conjugated anti-CD3 (1:100) antibodies and anti-rat IgG (1:100) under protection from light. Cells were sorted with a FACS Verse flow cytometer (Becton Dickinson, Sunnyvale, CA, USA). Data were collected for 10,000 events, stored in list mode, and subsequently analyzed.

Histological and immunohistological analyses

Hind paws and knee joints from BP-treated and untreated mice were dissected and fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS). Some specimens were processed for micro-computed tomography (CT) analysis as described below. After decalcification with 10% EDTA, specimens were dehydrated in a graded series of ethanol followed by xylene, then embedded in paraffin. Sections (5 µm in thickness) were stained with hematoxylin and eosin. Some sections were stained with toluidine blue at pH 4.1 to detect cartilage. The other paraffin sections were processed for the detection of tartrate-resistant acid phosphatase (TRAP) activity as previously indicated [41].

A subset of specimens were immersed in 5%, 15%, and 30% sucrose, embedded in Tissue Tek OCT compound (Sakura Finetek USA, Torrance, CA, USA), and fresh frozen in a mixture of acetone and dry ice for immunohistochemistry. Frozen sections (10 µm in thickness) were cut and mounted on poly-L-lysine-coated glass slides and air dried. After treatment with 0.3% H₂O₂/methanol for 30 min, sections were incubated with 5% normal goat serum in PBS containing 5% bovine serum albumin and 0.025% Triton X-100, followed by incubation with each of the monoclonal antibodies. After several rinses, the sections were incubated with biotinylated goat anti-rat or anti-hamster IgG followed by the avidin-biotin/horseradish peroxidase complex. After washing, sections were incubated in a mixture of 3,3’-diaminobenzidine tetrahydrochloride (0.5 mg/ml) (Wako Pure Chemical Industries, Osaka, Japan) and H₂O₂ at a final concentration of 0.03% in 0.1 M Tris-HCl buffer (pH 7.6). Sections were counterstained with methyl green. As a control, sections were incubated with either normal rat serum or PBS instead of the primary antibody.

Micro-CT analysis

The destruction of articular cartilage and bone was imaged using the SMX90-CT Micro Focus X-ray system (Shimazu Co., Kyoto, Japan). Imaging conditions were as follows: 87 kV, 16 µA, voxel resolution = 13 µm, and 1,200 steps. Imaging data were reconstructed, and the final 3-dimensional data had an area of 100 × 100 mm and a resolution of 45 µm/pixel. Data were reconstituted using a 3-dimensional imaging system (TRI-3D BONE; Ratoc Co., Tokyo, Japan).

Reverse transcription PCR

The synovium was dissected from knee joints under a microscope and immersed in RNA Later (Sigma-Aldrich Japan, Tokyo, Japan). Total RNA was extracted from each sample using an RNeasy Universal kit (Qiagen, Tokyo, Japan) and reverse transcribed (25°C for 10 min followed by 42°C for 12 min) to cDNA using Prime Script II 1st Strand cDNA Synthesis kit (Takara Bio, Tokyo, Japan); this was used as a template for
Table 1. Primer pairs used in this study

| Primer Pairs | PCR amplification of β-actin, IL-1β, TNF-α, IL-17, MMP3, MMP9, and MMP13 transcripts using specific primers (listed in Table 1). The reaction conditions were as follows: 95°C for 5 min; 30 cycles (40 cycles for IL-17) of 95°C for 10 s, annealing (at specific temperatures indicated in Table 1) for 30 s, and 72°C for 60 s; and 72°C for 7 min. PCR products were visualized on a 2% agarose gel stained with ethidium bromide using an E-box (Vilber Lourmat, Collégien, France).

Quantitative real-time (q)PCR

qPCR was carried out on a 7500 Fast Real-Time PCR system using the TaqMan Gene Expression Assay (both from Applied Biosystems, Foster City, CA, USA). Fold increases in mRNA transcript levels were calculated as follows: change in cycle threshold (ΔCt) = Ct (gene of interest) − Ct (β-actin), ΔΔCt = ΔCt CIA w/MIN group − average ΔCt CIA w/o MIN group, and fold difference = 2^{−ΔΔCt}. Values were normalized to β-actin.

Statistical analysis

Differences between groups were evaluated using the Student's unpaired t test or Mann-Whitney U test. P values < 0.01 were considered statistically significant.

Results

MIN increases arthritic score as well as serum TNF-α and MMP3 levels

MIN-treated mice exhibited higher arthritic scores at each time point examined as compared to saline-treated controls (Fig. 1a). At 2 weeks after the second sensitization, the score increased significantly in the MIN group. Serum concentrations of TNF-α (Fig. 1b) and MMP3 (Fig. 1c) were also increased by MIN treatment at each time point (Fig. 1b); the levels remained elevated 8 weeks after the second sensitization in the MIN group, in contrast to saline-treated control mice, which showed a rapid decline in the TNF-α level at this time point.

MIN inhibits bone resorption by osteoclasts

Given that MIN is an anti-bone resorption agent, we examined the effect of MIN on growth plate trabecular bones where many osteoclasts are attached to the bone surface and actively resorb bone. The volume of trabecular bone in the growth plate region was markedly increased by MIN treatment (Fig. 2a–c), indicating that MIN strongly inhibits physiological bone resorption by osteoclasts. TRAP-positive osteoclasts were detected along the trabecular bones in all groups (Fig. 2d–f). The intensity of TRAP activity was decreased by MIN-treatment (Fig. 2f).

MIN stimulates infiltration of inflammatory cells into arthritic joint cavities

Severe arthritis was induced in the joints of CIA mice, as evidenced by cartilage erosion and bone destruction observed by micro-CT analysis (Fig. 3a–c). Under normal conditions, synovial cells face the joint cavity surrounded by loose connective tissue (Fig. 3d); at week 8 after saline treatment, synovial cell proliferation with pannus formation, and inflammatory cell infiltration into the joint cavity were decreased (Fig. 3e). In contrast, pannus formation and massive inflammatory cell infiltration were maintained in MIN-treated CIA mice (Fig. 3f). An immunohistochemical analysis indicated that the majority of cells in the joint cavity were CD11b+Gr-1+ neutrophils (data not shown). Interestingly, necrosis was induced in bone marrow in the distal articular region of the femur by MIN treatment (Fig. 3f). Toluidine blue staining of the joints also indicated severe erosion of articular cartilage in both CIA groups compared to normal mice (Fig. 3g–i).
Figure 3. Morphological analysis of joint regions of normal, CIA without MIN and CIA with MIN at week 8 after the second sensitization. (a-c) Micro-CT. (d-f) Hematoxylin-Eosin staining. (g-i) Toluidine blue staining of articular cartilage. MIN enhanced pannus formation and the destruction of articular cartilage. Bar = 1mm (a-c), 200μm (d-f), 100μm (g-i).

Only a small number of neutrophils were detected in the joint cavity of normal mice (Fig. 4a, b); however, neutrophil accumulation was increased upon induction of arthritis (Fig. 4c–h). In the CIA w/o MIN group, significant neutrophil infiltration was observed at week 2 and 4 after treatment (Fig. 4c, d), although it was no longer detected at week 8 (Fig. 4e). In contrast, the massive infiltration of neutrophils persisted at 8-week time point in MIN-treated animals (Fig. 4f–h).

Figure 4. Flow cytometric analysis of infiltrating cells into the joint cavity. (a) Normal mice. (b) normal mice with MIN. (c-e) CIA without MIN. (f-h) CIA with MIN at week 2 (c, d), 4 (d, g) and 8 (e, h) after second sensitization. Almost all cells in the cavity were CD11b+ and Gr-1+ neutrophils.

IL-17 in the synovium has been implicated in the progression of arthritis. Since the main cell type producing IL-17 are Th17 and TCRγδ+ T cells, we examined whether these were among the cells invading into the synovium. A few number of CD4+ and TCRγδ+ cells were present in the synovium of CIA mice, but there were more CD4+ cells in the MIN (Fig. 5b, d) as compared to the saline group (Fig. 5a, c). At week 8, TCRγδ+ T cells were detected in the synovium of MIN treated mice (Fig. 5f) but not of saline group (Fig. 5e). The main cells infiltrated into synovium expressed CD4 (Fig. 5i) at week 4 whereas TCRγδ+ cells were predominant at week 8 (Fig. 5j).

MIN enhanced cytokine but not MMP levels in the synovium of arthritis mice

MMPs were continuously expressed in the synovium of normal mice and this was unaltered by MIN treatment (Fig. 6a). In contrast, MMP expression was strongly induced in the synovium in arthritic mice, although the levels were unaltered by MIN treatment. The expression of MMP9 seemed to decrease at week 8 by MIN treatment (Fig. 6a).

Meanwhile, cytokine levels in the synovium were also increased in CIA mice (Fig. 6b); the expression of IL-1β, TNF-α, and IL-17 was elevated at each time point examined. IL-1β expression was upregulated at week 2 and TNF-α expression was increased at week 4 and 8 after the second sensitization by MIN treatment, as determined by qPCR (Fig. 6c).

Figure 5. Immunohistochemical detection of CD4+ cells and TCRγδ+ cells in synovium. (a, c, e) CD4+ cells in synovium of CIA without MIN at week 4 (a) and 8 (c) and with MIN at week 4 (b) and 8 (d). TCRγδ+ cells in the synovium at week 8 of CIA without MIN (e) and with MIN (f). Negative control of CD4+ cells (g) and TCRγδ+ cells (h). Bars = 20μm. The changes of cell number of CD4+ cells of CIA without MIN and CIA with MIN (i) between week 4 and 8 and TCRγδ+ cells CIA with MIN (j) at week 8.

Discussion

In this study, we found that MIN exacerbated the severity of inflammation in arthritic mice by inducing the expression of pro-inflammatory cytokines, as we previously reported using ALN [35, 36]. A significant difference of clinical score was only detected between two groups at week 2. Inflammation of the joints was conspicuously progressed...
MIN induced bone marrow necrosis only in the distal femur; this effect was not observed in CIA treated with ALN. It has been proposed that the suppression of cholesterol synthesis by NBPs, secondary to FPP synthase inhibition, is causally related to the induction of necrosis [38, 43], which may explain in part the induction of bone marrow necrosis in this study. However, the mechanism underlying the tissue-specific induction of necrosis by MIN remains to be elucidated.

In conclusion, MIN exacerbated arthritis in a manner similar other NBPs by promoting the production of inflammatory cytokines and the infiltration of IL-17-producing cells into the synovium. However, the slight bone destruction and bone marrow necrosis resulting from MIN treatment suggests functional differences compared to other NBPs.

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