

Original

Direct Bone Destruction by Neutrophils in Collagen-induced Arthritis Treated with Bisphosphonates

Mayuko NAKAYAMA^{***}, Hideki YAGI^{***}, Yasuo ENDO^{****},
Koutaro MAKI^{**} and Masanori NAKAMURA^{*}

**Department of Oral Anatomy and Developmental Biology, Showa University School of Dentistry
1-5-8 Hatanodai, Shinagawa-ku, Tokyo, 142-8555 Japan*

***Department of Orthodontics, Showa University School of Dentistry
2-1-1 Kitasenzoku, Ohta-ku, Tokyo, 145-8515 Japan*

****Cell Biology Laboratory, Kinki University School of Pharmaceutical Science
Higashi Osaka, Osaka, 577-8502 Japan*

*****Department of Molecular Regulation, Tohoku University Graduate School of Dentistry
2-1 Seiryō-cho, Aoba-ku, Sendai, Miyagi, 980-8575 Japan*

Abstract: To identify the direct destruction of bone by neutrophils in collagen-induced arthritis treated with powerful anti-osteoclastic bone resorptive agents, bisphosphonates, we designed the experiments to examine whether the bone destruction induced by collagen-induced arthritis (CIA) was inhibited by nitrogen-containing bisphosphonates (NBPs) or not, and examined histologically and ultrastructurally to detect the cells directly related to bone destruction. One of the representative NBPs, alendronate (1.6 $\mu\text{mol/kg}$), was injected once a week from one week before the onset of the first sensitization with type II collagen. Flow cytometric analysis indicated the enhanced granulopoiesis in both BP-treated and non-treated CIA mice. Severe bone destruction was detected in both saline-group and alendronate-group. Although many active osteoclasts were developed on the destructive bone in saline-group, almost no active osteoclasts were detected in alendronate-group, where many neutrophils were accumulated. Some of neutrophils accumulated on the destructive bone were ruptured and cytoplasmic granules were scattered extracellularly, where collagen fibers were no longer detected. These results indicated that the bone destruction in CIA mice was not prevented by the injection of NBP and suggested that neutrophils in addition to osteoclasts might directly have the capacity for the bone destruction.

Key words: collagen-induced arthritis, bone destruction, neutrophil, osteoclast, bisphosphonate.

It has been well established that osteoclasts are the main cells for bone resorption.^{1, 2)} Until now, many studies were performed to clarify the mechanisms of bone resorption by osteoclasts and of cell differentiation of osteoclasts. Based on these results, new therapies to regulate the bone destruction in bone diseases have been also developed.^{3, 4)} However, it has been still controversial whether the cells other than osteoclasts destruct bone or not.

Bisphosphonates (BPs) are analogues of pyrophosphate with a potent inhibitory effect on bone resorption by osteoclasts.^{5, 6)} They have been used as

therapeutic agents in many bone destruction disorders, such as Paget's disease, metastatic bone diseases, malignant hypercalcemia, and osteoporosis.^{7~9)} Many derivatives of BPs have been developed by the modification of the chemical residues of carbon. Among these derivatives, nitrogen-containing BPs (NBPs) is most powerful agents to inhibit bone resorption.^{10, 11)} Alendronate is one of the typical BP. Our previous study indicated that a single injection of alendronate at the dosage of 1.6 mmol/kg inhibit osteoclastic bone resorption for 1 week.¹²⁾

Rheumatoid arthritis (RA) is characterized by

chronic inflammation of synovium, progressive pannus formation and ultimate destruction of bone and cartilage.^{13~15)} The pathologic destruction of bone and cartilage has been generally considered to be the result of elevated osteoclastogenesis and consequent enhancement of bone resorption by the increased number of osteoclasts in the affected joints. Recently, in parallel with these destructive changes in RA, unusual myeloid cells bearing an oncofetal surface marker were recognized in the bone marrow of the RA patients.^{16~18)} Accordingly, possible role(s) of these unusual granulocytes in the progression of RA have been speculated.

In our previous study, we reported a significant increase in the percentage of immature neutrophils in the bone marrow of RA patients, and that this increase well correlated with the severity of the disease (Lansbury index score).¹⁹⁾ Careful ultrastructural observation revealed that abundant neutrophils accumulated on the surface of bone trabecula of the iliac bone, that some of these cells were ruptured and their cytoplasmic granules were scattered close to bone surface, and that the collagenous fibers in the bone matrix disappeared from those bone spicules.¹⁹⁾ Alternate bone destruction by cells other than osteoclasts was thus strongly implicated. This type of bone destruction would be entirely different from the conventional bone resorption by osteoclasts.^{1, 20~22)} Neutrophils have many types of proteolytic enzymes in their cytoplasmic granules²³⁾ and are reported to play a major role for the degradation of extracellular matrix in the inflamed tissue.^{24~27)} Collectively, these results strongly suggest the possible bone destruction by neutrophils.

In this study, to confirm the hypothesis that neutrophils could degrade bone matrix in a more direct way, we examined whether bone destruction in collagen-induced arthritis (CIA) was prevented by BP treatment and the possibility of bone destruction by neutrophils.

Materials and Methods

The experimental protocol used was reviewed and

approved by the Animal Care Committee of Showa University.

Mice and reagents

Male DBA/1 mice (6 weeks old) raised under specific pathogen-free conditions purchased from Saitama Breeding Laboratory, Saitama, Japan. Bovine type II collagen prepared by Collagen Research Center (Kiyose, Japan) was purchased from Cosmo-Bio Inc., Tokyo, Japan.

The nitrogen-containing bisphosphonate, alendronate, was synthesized by ourselves.¹²⁾ Alendronate was dissolved in sterile saline and their pH adjusted to 7 with NaOH. This solution was injected intraperitoneally (i.p., 0.1 ml per 10 g body weight).

Biotinylated monoclonal antibodies, Gr-1, and non-labeled Mac-1 were purchased from PharMingen, San Diego, CA. Fluorescent isothiocyanate (FITC)-labeled goat anti-rat IgG antibody and streptavidin-FITC were purchased from Jackson ImmunoResearch Lab., West Grove, PA.

Induction of collagen-induced arthritis

Arthritis was induced in DBA/1 mice by the injection of the mixture of type II collagen and Freund's complete adjuvant. The emulsion consisted of 1 ml of 0.2% type II collagen in 0.01 M acetic acid and 1 ml of the adjuvant were made by mixing homogenously with the sonicator and 50 μ l of the mixture was injected intradermally at the root of the tail of DBA/1 mice of 7 weeks old age (first sensitization). Three weeks later, the same mixture was again injected in the same way (second sensitization).

The severity of the joint inflammation in each mouse was quantified by scoring each paw on a scale of 0 to 4. The incidence of arthritis and total of the score for all four paws were used as indices of the arthritis produced. At 8 weeks after the second sensitization, mice were killed and processed for the following experiments.

Administration of bisphosphonates

Alendronate (1.6 μ mol/kg) was i.p. injected once per week from 1 week earlier than the sensitization to

eliminate the possibility of bone resorption by osteoclasts. In the control group, same volume of saline was i.p. injected at the same intervals.

Cell preparation and flow cytometry of bone marrow cells

Mice were perfused with Dulbecco's phosphate-buffered saline (PBS) from the aorta abdominals for 90 s under Nembutal anesthesia.

The femurs were dissected and the ends of femurs were cut off with scissors. Single cell suspensions of the bone marrow were prepared by flushing the marrow with a staining medium (RPMI 1640 [Irvine Scientific, Santa Ana, CA] containing 10 mM Hepes, 3% FCS, and 0.1% NaN₃) using a 1.0 ml syringe and 26-gauge needle.

Cells were incubated on ice with optimal amount of FITC-labeled, biotin-conjugated or non-labeled mAbs for 45 min and washed three times with the staining medium. Biotin-conjugated mAbs and non-labeled mAbs were developed with phycoerythrin (PE)-streptavidin and FITC-goat anti-rat IgG antibody, respectively. Finally, cells were analyzed on a FACS Calibur flow cytometer (Becton and Dickinson, Sunnyvale, CA).

Data were collected for 10,000 events, which were stored in a list mode, and subsequently analyzed using the CellQuest software (Becton Dickinson). Analysis of cell samples always included propidium iodide (1mg/ml) for dead cell exclusion. Mature erythrocytes were excluded using forward and side scatter parameters.

Histological observation of the bone destruction

Hind paws and knee joints from BP-treated and non-treated mice were dissected and fixed with 4% paraformaldehyde in PBS. After the decalcification with 10% EDTA, specimens were dehydrated with the graded series of ethanol, passed through xylene and embedded in paraffin. Five mm thick sections were made and stained with hematoxylin and eosin. Some of the sections were processed for the detection of tartrate-resistant acid phosphatase (TRAP) activities described elsewhere.²⁸⁾

For transmission electron microscopy, specimens were fixed with the mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). After the decalcification with 10% EDTA, specimens were post-fixed with 2% osmium tetroxide, dehydrated with the graded series of ethanol, passed through propylene oxide and then embedded in EPON 812. Ultra-thin sections were stained with uranyl acetate and lead citrate and observed with a Hitachi H-7500 electron microscope (Hitachi, Tokyo, Japan).

Results

Effect of alendronate on arthritis

Figure 1 showed the effect of alendronate on collagen-induced arthritis. Compared with the saline-group, the alendronate-group showed a higher arthritic score at every time point. However, no significant differences were detected between two groups.

Flow cytometric analysis of hematopoiesis in arthritis

To simplify the analysis, we used mAbs Gr-1 and Mac-1 to identify granulocyte lineage cells and monocyte-macrophage lineage cells. Gr-1-positive and Mac-1-positive (Gr-1⁺/Mac-1⁺) cells, and Gr-1-negative and Mac-1-positive (Gr-1⁻/Mac-1⁺) cells were identified as granulocyte lineage cells and monocyte-

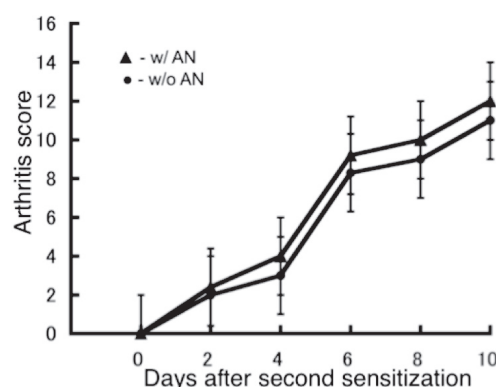


Fig. 1 Arthritis score of CIA. The saline-group (w/o AN) and the alendronate-group (w/AN) presented the tendency for inflammation to get aggravation after two week of secondary sensitization. No significant differences were detected between two groups.

macrophage lineage cells, respectively. The major population of granulocyte lineage cells are neutrophil lineage cells.

Forward- and side-scatter analysis revealed an increase of granulocyte population in bone marrow in arthritis (Fig. 2). Consistent with this finding, a marked increase of Gr-1⁺/Mac-1⁺ cells was detected in arthritis mice. By alendronate treatment, the ratio of Gr-1⁺/Mac-1⁺ cells was slightly increased (from 39.9% [saline-group] to 41.2%), however, no significant differences were detected between two groups.

Histological analysis of joints in arthritis mice

In both groups, severe arthritis was observed in the joints. Severe synovial proliferation with pannus formation, cartilage erosion and massive infiltration of inflammatory cells into the joint were evident in both groups (Fig. 3A, B). Marked bone destruction around the joint was also detected in saline-group (Fig. 3A). TRAP staining indicated that many osteoclasts were located along the bone surface and resorbed bone in saline-group (Fig. 3C), which indicated the active bone resorption and destruction by osteoclasts. Severe bone destruction was also evident in alendronate-group (Fig. 3B). However, only a few osteoclasts were detected at bone destructive area. Furthermore, these osteoclasts

were located apart from the bone surface (Fig. 3D). Many polymorphonuclear cells were detected on the surface of destructive bone (Fig. 3D).

The effect of alendronate on bone resorption in the growth plate of tibias was shown in Fig. 4. The number of bone trabecula at the growth plate was markedly increased in the alendronate-group (Fig. 4B) compared to saline-group (Fig. 4A). These results indicated that alendronate strongly inhibited the physiological bone resorption mediated by osteoclasts.

Ultrastructural detection of bone destruction in alendronate-group

In severe bone destruction area, the surface of bone showed irregular margin where many granulocytes were accumulated (Fig. 5A, Fig. 6A). In addition to ragged margins, obviously destructive alteration of the bone could be observed. Some of the granulocytes adhering to the bone were in a degradation process (Fig. 6A). No cell membrane could be detected and small electron dense granules were scattered extracellularly (Fig. 6B). Furthermore, bone-lining cells could not be detected around these destructive bone surfaces (Fig. 5A, Fig. 6A).

The collagenous fibers in surface layer of bone matrix became obscure or still observed in the matrix

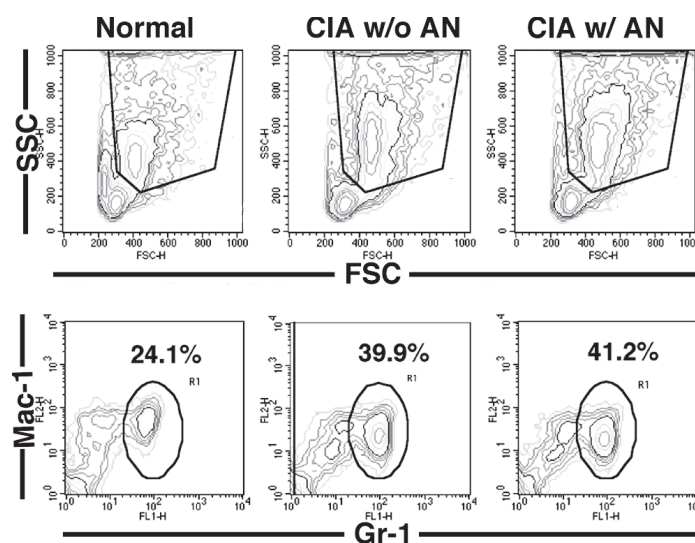


Fig. 2 Flow cytometric analysis of bone marrow. Upper layer showed the FSC and SSC analysis of normal, saline-group (w/o AN) and alendronate-group (w/ AN). Lower layer indicated the two-color analysis of bone marrow cells by Gr-1 and Mac-1 antibodies. Enhanced granulopoiesis was evident in both saline- and alendronate-groups. No significant differences were detected between two groups.

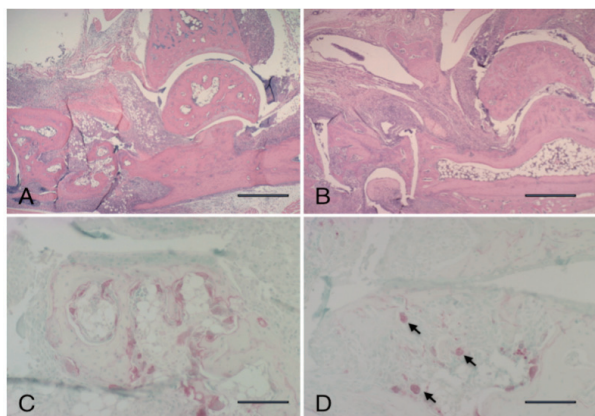


Fig. 3 Histological detection of bone destruction of hind paw joints of saline-group (A, C) and alendronate-group (B, D). Haematoxylin-eosin staining (A, B). TRAP staining (C, D). Pannus formation, cartilage erosion and bone destruction were evident in both groups (A, B). (C) Many osteoclasts were detected at the destructive bone surface. (D) Osteoclasts were removed from the bone surface (arrows) and many polymorphonuclear cells were accumulated on the bone surface. Bar scale=250 μ m.

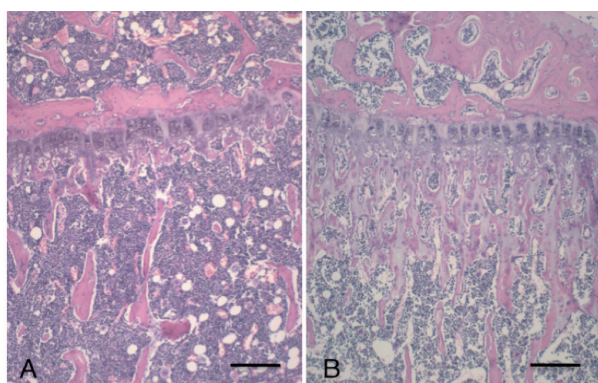


Fig. 4 Histological observation of tibial growth plates from arthritis mice of saline-group (A) and alendronate-group (B). A marked increase in trabecular bones was evident in the growth plate of alendronate-group, indicating a strong inhibition of osteoclast-mediated bone resorption. Bar scale=200 μ m.

at the superficial layer. However, these fibers were apparently thinner than those in the deep layer and evidently much more sparsely arranged (Fig. 5B). In some bone matrix adhering to the degrading neutrophils, collagenous fibers could not be detected (Fig. 6B).

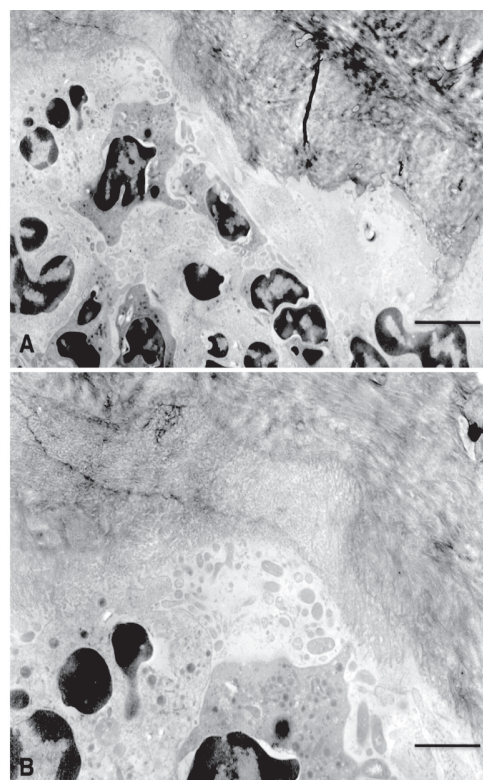


Fig. 5 Electron micrographs of destructive bone of alendronate-group. (A) Many neutrophils were accumulated on the bone surface which showed an irregular margin. No bone-lining cells were observed. (B) Higher magnification of destructive bone matrix. Coarsely distributed collagenous fibers were evident. Bar scale=5 μ m.

Discussion

The bone destruction in collagen-induced arthritis was not prevented by alendronate treatment in this study. In saline-group, massive bone destruction was detected which mediated by osteoclasts. On the contrary, almost few osteoclasts were localized on the bone surface in alendronate-group. Furthermore, the resorption of growth plate bone trabecula was critically inhibited by alendronate treatment. These results strongly suggested that the cells other than osteoclasts might destruct bone.

In alendronate-group, flow cytometric analysis indicated the enhancement of granulopoiesis (Gr-1⁺/Mac-1⁺ cells) in the bone marrow, which was confirmed by the ultrastructural observation showing the massive accumulation of neutrophils on the bone surface. These

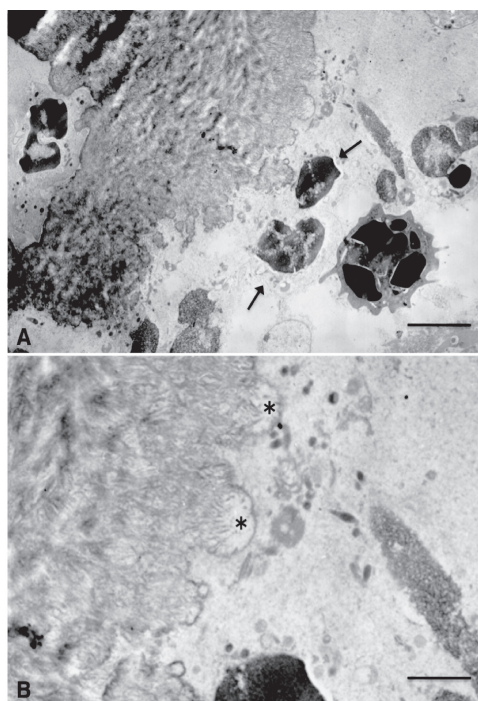


Fig. 6 Electron micrographs of destructive bone of alendronate-group. (A) Neutrophils on the bone surface lost the cell membrane (arrows) and the bone surface showed an irregular margin. (B) Higher magnification of destructive bone. Disappearance of collagenous fibers in the bone matrix (*) and the extracellularly dispersed cytoplasmic granules were evident. Bar scale=5 μ m.

results were well coincident with the results of severe RA patients progressing bone destruction as indicated by Ohtsu et al.¹⁹⁾ In human RA, unusual myeloid cells expressing an oncofetal surface marker were detected in the bone marrow and suggested the possible role of these cells for the disease progression.^{16~18)} As further characterization of neutrophils in alendronate-group was not investigated in this study, there might be a possibility that neutrophils in alendronate-group bound unusual phenotypes.

In alendronate-group, bone-lining cells were not detected around the destructive bone surface. Bone-lining cells usually covered the bone surface except for the active bone resorption sites by osteoclasts. Bone lining cells were also the member of barrier cells in the bone marrow.²⁹⁾ These cells functioned as the supporting cells for hematopoiesis and the barrier to protect the expansion of microenvironment

damage induced by enhanced hematopoiesis. Our results that the disappearance of bone-lining cells and the accumulation of neutrophils on the bone surface suggest the disruption of the bone marrow microenvironment which might be the key event for the bone destruction by neutrophils.

Neutrophils have several types of cytoplasmic granules that contain many kinds of proteolytic enzymes.²³⁾ Some of these enzymes like elastase and MMPs are able to degrade extracellular matrix.^{24, 30)} Therefore, neutrophils have been regarded as one of the major cells responsible for the destruction of soft tissues^{25, 26, 31)} and articular cartilages in RA.^{27, 32)} Until our previous study, however, no other study had ever suggested neutrophils as effector cells for bone destruction in RA. The remarkable observation of our previous study was that neutrophils, by rupturing whole cells by themselves, disseminated cytoplasmic granules over and within the bone matrix. Accordingly, at the site of granule dissemination, collagen fibers disappeared to a greater extent from the bone matrix. Okada et al. demonstrated that MMP-9 could degrade collagenous fibers in decalcified bone fragments.³³⁾

Although we did not analyze the mineral release from the bone matrix by neutrophils, several *in vitro* studies so far demonstrated the significant increase of the amount of Ca release from the bone by neutrophils.^{34, 35)} Furthermore, Kleter et al. indicated that the decalcification of dentin was accelerated when the matrix was degraded by the alternate incubation of dentin with acid solution and collagenase.³⁶⁾ All these results support the hypothesis that neutrophils can concurrently decalcify bone matrix mineral as well as degrade collagen fibers in the bone matrix.

In conclusion, we confirmed in this study that (1) bone destruction in CIA was not improved by bisphosphonate, (2) neutrophils were accumulated on the destructed bone surface, (3) disappearance or ultrastructural alteration of collagen fibers were detected in the destructed bone matrix. Our results that neutrophils could degrade bone matrix might help to

develop a new therapeutic procedure to treat RA with progressive bone destruction.

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