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ORIGINAL ARTICLE

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Minodronic acid influences receptor activator of nuclear factor κB ligand expression and suppresses bone resorption by osteoclasts in rats with collagen-induced arthritis

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Abstract We investigated the inhibitory mechanism of bone resorption by minodronic acid in collagen-induced arthritis (CIA) in rats. Four groups of female Sprague–Dawley rats, aged 7 months, were studied: three groups of collagen-sensitized rats, including one placebo-administered group (CIA-P), and two minodronic acid-administered groups at 0.2 mg/kg/2 day (CIA-BIS) and 2.0 mg/kg/2 day (CIA-BIS10). These were studied with an additional untreated observation group (Cont group). Minodronic acid was administered orally a day after the initial sensitization. The femoral posteromedial condyle was analyzed histologically and immunohistologically 4 weeks after the initial sensitization. Western blotting was also performed to assess the receptor activator of nuclear factor κB (RANK), RANK ligand (RANKL), and osteoprotegerin (OPG) expression of the knee joints. In CIA-P rats, many tartrate-resistant acid phosphatase (TRAP)-positive cells were found at the pannus-lining layer and the epiphyseal medulla. The bone-lining cells in the epiphyseal medulla and the cells in the pannus strongly expressed RANK and RANKL. In the minodronic acid-administered group, the number of TRAP-positive cells and the severity of arthritis were reduced. The reduction in the CIA-BIS10 group was significant compared with the CIA-P group ($P < 0.05$). Dosage-dependent reduction of RANK and RANKL expression was confirmed by immunohistology and Western blotting. With or without minodronic acid administration, no apoptotic cells were found in any groups using the TdT-mediated dUTP-biotinnick end labeling (TUNEL) method. The expression of OPG was not clear in all groups. These results demonstrated that minodronic acid inhibited the differentiation and the activation of osteoclasts not by inducing apoptosis but by inhibiting the RANKL–RANK system, and thereby suppressing bone resorption.

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Introduction

Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic synovitis, and is known to cause generalized and periarticular bone loss. In 1997, Trentham et al.[1] reported that sensitization to type II collagen caused arthritis (collagen-induced arthritis, CIA) similar to RA. CIA has been used as a RA model for studies on the pathology and treatment of RA. We demonstrated that the mature CIA rat is a useful model of periarticular bone loss in RA[2] and have investigated the effects of various drugs and mechanical stress on bone loss in CIA.[3–5]

Bisphosphonates are agents with an inhibitory effect on bone resorption, which maintains and increases the bone mass. Minodronic acid, a nitrogen-containing bisphosphonate, is an agent that has an amino group on the imidazole ring and has a potent inhibitory effect on bone resorption.[3] We reported the inhibitory effects of this agent on arthritis, as well as its superiority in maintaining bone mass.[3] The nitrogen-containing bisphosphonates inhibit osteoclastic bone resorption owing to inhibition of the mevalonate pathway.[6] However, this mechanism cannot explain the anti-inflammatory effects of minodronate. The mechanism of bisphosphonates is poorly understood. The objective of this study was to elucidate the inhibitory mechanism of bone resorption by minodronic acid in CIA rats using histological/immunohistochemical methods.

Materials and methods

Animals

Seven-month-old female Sprague–Dawley rats [retired breeding animals, body weight (BW) 255–355 g, Shimizu

Laboratory Supply, Kyoto, Japan] were used in the experiment. This experiment was carried out in accordance with the Guidelines for Animal Experimentation of the Faculty of Medicine, Tottori University. During the experiment, animals were freely fed with tap water and food (CE-2; CLEA, Japan, Tokyo; calcium content 1.18 g/100 g, phosphorus content 1.09 g/100 g, vitamin D3 content 250 IU/100 g). The room temperature was set to 24°C, and after about 2 weeks of preliminary breeding, the animals were used for the experiments. The animals were divided randomly into four groups: three groups of collagen-sensitized rats (each group $n = 4$), including one placebo-administered group (CIA-P) and two minodronic acid-administered groups at 0.2 mg/kg/2 day (CIA-BIS) or 2.0 mg/kg/2 day (CIA-BIS10). These were studied with an additional untreated observation group ($n = 4$, Cont group).

Collagen arthritis models

According to the method described by Trentham et al.,[1] 1 ml of emulsion containing 0.5 mg bovine type II collagen (0.3% acetic acid solution; K-41 Cosmo-Bio, Tokyo, Japan) and 0.5 mg of incomplete Freund's adjuvant (521-00021; Difco Laboratories, Detroit, MI, USA) were injected intracutaneously at three sites on the back of CIA group rats ($n = 12$), and one week later, a half dosage of the same emulsions was injected. In the Cont group, physiological saline was injected in the same manner. The development of arthritis was judged by the redness and swelling in the ankle by the same observer.

Minodronic acid administration

Minodronic acid (ONO-5920/YM529; chemical name [1-hydroxy-2-(imidazo[1,2-*a*]pyridine-3-yl)etylidene]-bisphosphonic acid monohydrate) was dissolved in 1 N NaOH solution and mixed with 2% methylcellulose. Methylcellulose solvent alone was administered orally to the CIA-P group. Administration was started orally 1 day after the initial sensitization. The frequency of administration was three times a week, and the animals were fasted for 2 h before and after the administration.

Evaluation of arthritis

Body weight, arthritis score,[7] and width of the hindlimb were measured each week. The widths of the left and right hindlimbs were compared, and the one with larger swelling was used. The arthritis scores were evaluated as 0–4 points for one limb (0 = no arthritis, 1 = redness in at least one or more joints, 2 = inflammation in at least two or more joints or moderate inflammation in one or more joints, 3 = severe inflammation in one or more joints, and 4 = severest inflammation in one or more joints) and were summed up (maximum score 16 points).

Histological evaluation

The rats were killed 4 weeks after the initial sensitization. The knee joint with larger swelling in each rat was collected with the joint capsule and synovial membranes preserved. After fixation with 4% buffered paraformaldehyde-phosphate-buffered saline (PBS, pH 7.4) at 4°C for 16 h, they were decalcified with 10% ethylenediaminetetraacetic acid (EDTA) in PBS (pH 7.4) for 4 weeks. After decalcification, they were paraffin-embedded. Serial tissue sections of the knee joint at 4 μm were prepared sagittally with a microtome. After the tissue sections were dried at 54°C for 16 h on a silane-coated superfrost slide glass (Matsunami Glass Industries, Osaka, Japan), they were hematoxylin–eosin (H&E) stained and immunostained [enzyme antibody, Streptavidin-biotin (SAB) method]. The posteromedial femoral condyle was mainly observed.

TRAP staining

According to the method described by Suzuki et al.,[8] after washing the deparaffined tissue sections with PBS for 5 min, tartrate-resistant acid phosphatase (TRAP) staining was performed by immersing them in PBS supplemented with 1 mM magnesium and 1 mM calcium for 16 h to activate TRAP that was inactivated with EDTA, followed by washing with PBS for 10 min. Subsequently, they were stained with a reagent (Sigma Chemical, St. Louis, MO, USA) containing acetate buffer (pH 5.0), naphtol AS-BI phosphate, Fast Garnet GBC, and 50 mM sodium tartrate at 37°C for 1 h according to the method described by Fujikawa et al.[9] They were counterstained with Harris Hematoxylin solution (099H4396, Sigma Diagnostics, St. Louis, MO, USA). In randomly sampled 12 sections for each group, three sections per rat, TRAP-positive multinucleated giant cells present at the tips of the pannus, infiltrating the epiphyseal medulla of the posteromedial femoral condyle, were counted in ten arbitrary fields (400 × objective: E800M, Nikon ECLIPSE, Tokyo, Japan), and the total was calculated and compared among the different groups.

Immunohistological staining SAB method

Receptor activator of nuclear factor κB (RANK), RANK ligand (RANKL), and osteoprotegerin (OPG) immunostainings were performed according to the method described by Lubberts et al.[10] After washing the deparaffined tissue sections with PBS for 5 min, they were immersed in 3% hydrogen peroxide solution for 15 min to block the endogenous peroxidase. After washing with PBS for 5 min, blocking with 10% normal rabbit serum was performed at 37°C for 10 min. Subsequently, they were reacted with primary antibodies (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight: polyclonal anti-RANK (dilution ratio 1:100; sc-9072), anti-RANKL (dilution ratio 1:50; sc-9073), and anti-OPG antibodies (dilution ratio 1:100; sc-11383). The subsequent staining was performed using a Histofine SAB-PO kit (424132; Nichirei, Tokyo, Japan),

DAB (3.3-diaminobenzidine tetrahydrochloride), and a coloring kit (42501; Nichirei, Tokyo, Japan). On immunohistochemical examination of the femoral epiphyseal medulla and the pannus, the expression sites of RANKL, RANK, and OPG proteins during the onset of arthritis and their intensities were observed. The changes in expression with bisphosphonate administration were evaluated.

TUNEL method

To observe apoptotic osteoclasts, a TUNEL method (Apop TagOR Peroxidase In situ Apoptosis Detection Kit; Funakoshi, Tokyo, Japan) was performed. After the deparaffined sections were digested with proteinase K (20 μg/ml) for 15 min at room temperature, they were immersed in 3% hydrogen peroxide solution for 15 min to block endogenous peroxidase. After washing with PBS, they were incubated with biotin-labeled deoxyuridine 5′-triphosphate (dUTP)-containing terminal deoxynucleotidyl transferase (pH 7.2, 19 U/ml), 1 nM $CoCl_2$ buffer, 140 mM sodium cacodylate, and 30 mM Tris in a chamber at 37°C for 1 h. Subsequently, they were washed twice with 30 nM NaCl and 30 mM sodium citrate for 5 min. Nonspecific antigens were blocked by immersing them in 2% bovine serum albumin (BSA) for 10 min. The biotins were detected by immersing them in peroxidase-labeled streptavidin diluted 1 : 200 in 0.1 M Tris, 0.5 M NaCl, 0.01 M $MgCl_2$, and 0.05% Tween20 for 30 min. After washing with PBS, they were stained using a DAB coloring kit. They were counterstained with Harris Hematoxylin solution (099H4396, Sigma Diagnostics, St. Louis, MO, USA).

Western blotting

Western blotting was performed to quantitatively assess RANKL, RANK, and OPG expression. Rats were killed at 4 weeks after sensitization, and one rat was randomly assigned to each group. Knee joints were collected with joint capsules preserved, frozen in liquid nitrogen, and stored frozen. To extract proteins from the tissues, a mixer mill MM300 (Qiagen, Tokyo, Japan) was used. Briefly, samples were crushed with silicone beads in 1 ml of lysis buffer (50 mM Tris–HCl, pH 7.4, 125 mM NaCl, 0.1% NP-40, 5 mM NaF, 1 mM PMSF, 1 ng/ml leupeptin, 10 ng/ml soybean trypsin inhibitor, 1 ng/ml aprorinin, and 10 ng/ml *N*-tosyl-L-phenylalanyl chloromethyl ketone) twice for 10 min at 4°C with a mixer mill. The dissolved samples were centrifuged at 15 000 rpm for 10 min at 4°C, and the supernatants were diluted 1 : 500 in protein assay reagent (500-0114, Bio-Rad Laboratories, CA, USA). BSA was used as a control. Equivalent amounts of proteins (20 μg) were separated on a 12% polyacrylamide gel (sodium dodecyl sulfate–polyacrylamide gel electrophoresis, SDS–PAGE), and electrophoresed on a polyvinylidene difluoride thin membrane (Millipore, Bedford, MA, USA). Nonspecific antigens were blocked with 10% skim milk [solvent PBS (–)] at room temperature for 1 h. Subsequently, primary antibodies were reacted on the membrane. The following antibodies (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used as primary antibodies: polyclonal anti-RANK (dilution ratio 1 : 200; sc-9072), anti-RANKL (dilution ratio 1 : 200; sc-9073), and anti-OPG antibodies (dilution ratio 1 : 200; sc-11383). One hour later, secondary antibody (1 : 1000; MBL, Nagoya, Japan) was administered and reacted for 1 h at room temperature. Blotting was performed using an enhanced chemiluminescence (ECL) Western blotting kit (Amersham Pharmacia Biotech, Bucks, UK).

Statistical analysis

Nonrepeated measure analysis of variance (ANOVA) was utilized for comparative analysis among different groups. $P < 0.05$ was judged as a significant difference. When there was a significant difference, a Student–Newman–Keuls test was performed as a multiple comparison test. Statistical analyses were performed in Microsoft Excel 2003 for Windows.

Results

Changes in BW

No apparent weight change was seen in the Cont group. With or without minodronate administration, significant weight loss occurred in the CIA groups when compared with the Cont group (Fig. 1). There was no significant difference among the three CIA groups.

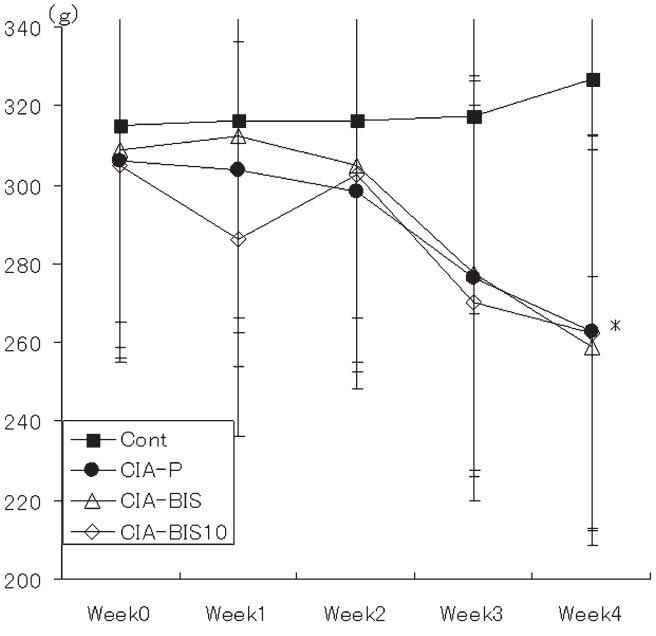

Fig. 1. Changes in body weight (BW). There was no BW change in the course in the Cont group. BWs of the sensitized groups [collagen-induced arthritis (CIA)-P, CIA-BIS, and CIA-BIS10] were significantly reduced compared with the Cont group (*$P < 0.05$)

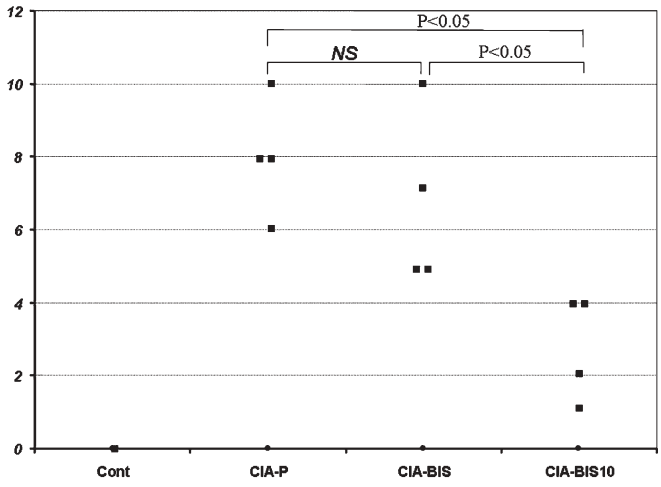

Fig. 2. Arthritis score at 4 weeks after the initial sensitization. The CIA-BIS10 group showed significantly lower values compared with CIA-P (*$P < 0.05$) and CIA-BIS (*$P < 0.05$) groups. There was no difference between the CIA-P and CIA-BIS groups

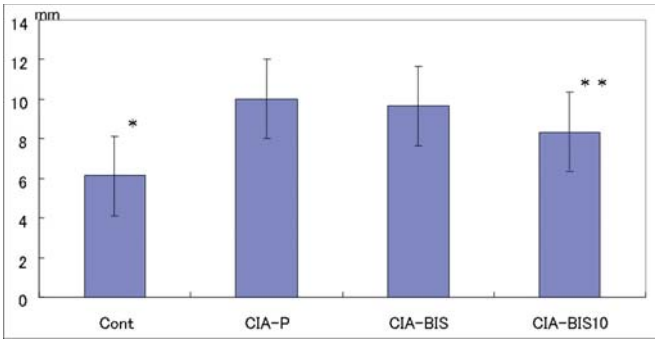

Fig. 3. Thickness of the hindlimb. There was a significant increase in the width of the hindlimb (those with larger swelling were measured; the graph shows the means of each group) in the groups at 4 weeks after the initial sensitization [CIA-P (*$P < 0.05$), CIA-BIS (*$P < 0.05$), and CIA-BIS10 (*$P < 0.05$)] compared with the Cont group. The increase in the width of the hindlimb in the CIA-BIS10 group was less compared with those in the CIA-P and CIA-BIS groups (**$P < 0.05$). The width of the hindlimb of each group is as follows. Cont group (6.1 mm, 6.3 mm 6.0 mm, 6.1 mm), CIA-P group (9.5 mm, 9.9 mm, 11.3 mm, 9.9 mm), CIA-BIS group (9.1 mm, 10.9 mm, 8.3 mm, 10.5 mm) CIA-BIS10 group (8.5 mm, 6.8 mm, 8.9 mm, 9.2 mm)

Arthritis score and the width of the hindlimb

In the 4-week observation of the Cont group, no change was seen in the joints of the extremities. The incidence of arthritis in the three CIA groups was 100% at 4 weeks after sensitization. Arthritis developed on day 10 after the initial sensitization. No difference in the onsets was noted among the groups. At 4 weeks after sensitization, the arthritis score of the CIA-BIS10 group was significantly lower than that of the other groups ($P < 0.05$; Fig. 2). Swelling of the hindlimb of the CIA-BIS10 group was significantly lower than that of the other two CIA groups ($P < 0.05$). There was no significant difference between the CIA-P and CIA-BIS groups (Fig. 3).

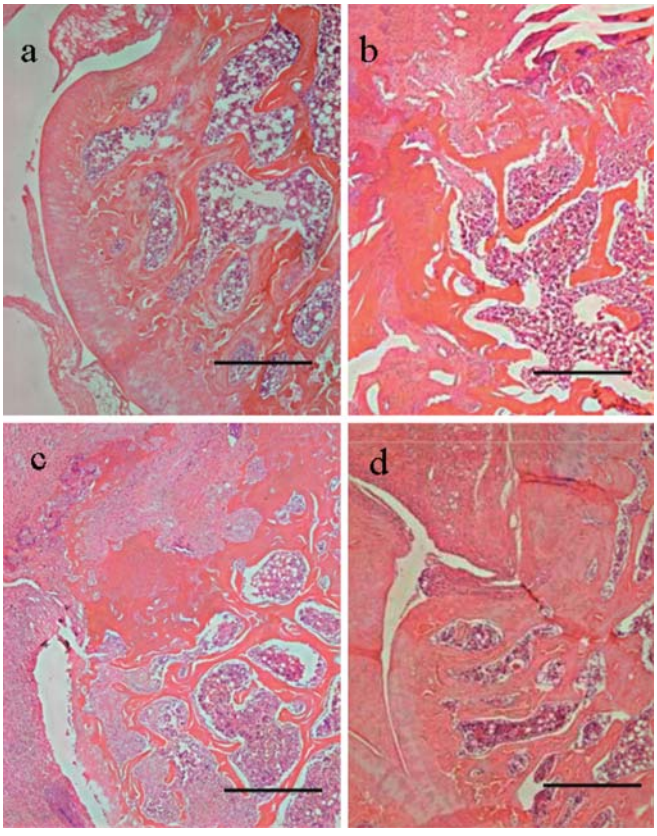

Fig. 4. Hematoxylin–eosin (H&E)-stained micrographs of the knee joint at 4 weeks after sensitization; *bar* 500 µm. **a** Cont group. **b** CIA-P group: pannus was found in the knee joint, infiltrating the femoral medulla. **c** CIA-BIS group: there was a smaller amount of the pannus in the CIA-BIS group than that in the CIA-P group; the bone destruction was mild. **d** CIA-BIS10 group: little pannus was found, and the structures of the bones and joints were preserved

Histological and immunohistological findings

The knee joints at 4 weeks after the initial sensitization were evaluated by H&E-stained specimens (Fig. 4). No femoral and tibial destruction was observed in the Cont group, nor was the pannus (Fig. 4a). In the CIA-P group, pannus proliferating from the bare area around the femoral articular cartilage was found, which destructed the surface of the femoral cartilage and caused erosion into the medulla (Fig. 4b). The pannus of the CIA-BIS group was slightly milder when compared with that of the CIA-P group (Fig. 4c). In the CIA-BIS10 group, pannus did not proliferate, and the joint structure was preserved (Fig. 4d).

The number of TRAP-positive cells

The knee-joint specimens at 4 weeks after the initial sensitization were TRAP-stained, and TRAP-positive cells on the pannus-lining layer infiltrating the posteromedial femoral condyle were counted (Fig. 5). The TRAP-positive cells in the CIA-BIS10 group decreased compared with those in the CIA-P and CIA-BIS groups ($P < 0.05$, Fig. 6). The TRAP-positive cells tended to decrease in the CIA-BIS

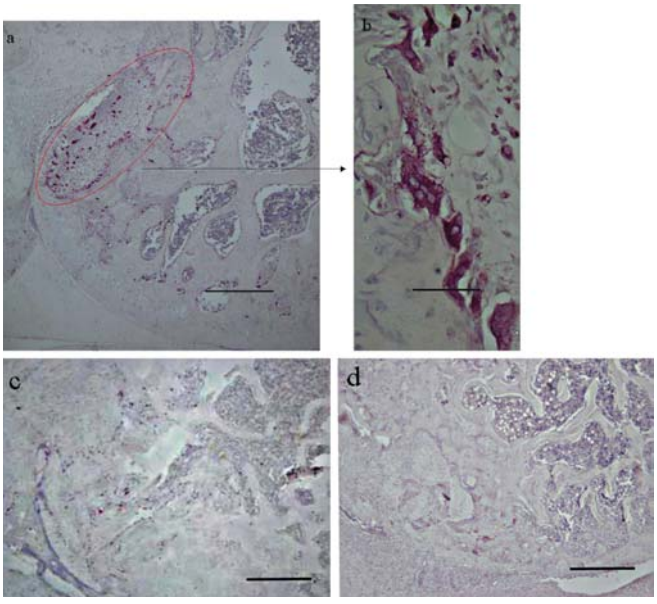

Fig. 5. Tartrate-resistant acid phosphatase (TRAP)-stained micrograph of CIA-P group. TRAP-positive multinucleated cells present at the tips of the pannus infiltrating the femur were counted. **a**, **b** CIA-P group, **c** CIA-BIS group, **d** CIA-BIS10. **a**, **c**, **d** *Bar* 500μm; **b** *bar* 50μm

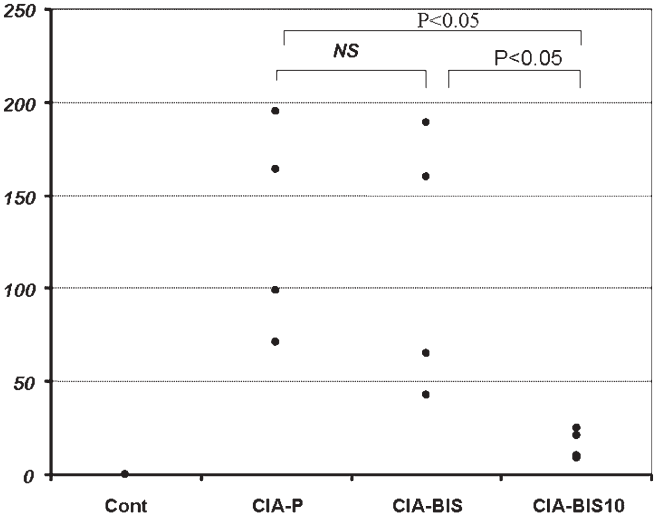

Fig. 6. Number of the TRAP-positive cells in each group. There were many TRAP-positive multinucleated cells in the CIA groups. There was a marked reduction in the CIA-BIS10 group compared with the CIA-P (*$P < 0.05$) and CIA-BIS (*$P < 0.05$) groups

group compared with those in the CIA-P group, but there was no significant difference. No TRAP-positive cells were found in the Cont group.

The number of TUNEL-positive cells

The knee joints at 4 weeks after the initial sensitization were evaluated by TUNEL-stained specimens. In the epiphyseal medulla of the femur in all groups, no TUNEL-positive cells were observed among the pannus-lining multinucleated giant cells.

RANK expression in the synovial membrane and the medulla

RANK was not expressed in the synovial membrane and the medulla of the Cont group (Fig. 7a). RANK was strongly expressed in the cells of the highly proliferating pannus in the CIA-P group and in the bone-lining cells of the femoral epiphyseal medulla where the pannus was infiltrating (Fig. 7b). However, the expression in the bone-lining cells distant from the pannus was weak, and little expression was found in the lining cells in the metaphyseal portion of the bone. In the CIA-BIS group, RANK protein expression was confirmed in the cells within the pannus and the bone-lining cells, but was lower compared with that in the CIA-P group (Fig. 7c). In the CIA-BIS10 group, there was little RANK protein expression both in the pannus and in the medulla (Fig. 7d). In all groups, no RANK protein expression was found in the lining cells of the metaphyseal and diaphyseal portions of the bone.

RANKL expression in the synovial membrane and the medulla

The expression pattern was similar to that of RANK protein (Fig. 8). In the Cont group, no RANKL protein expression was seen in the medulla (Fig. 8a). In the CIA-P group, RANKL was strongly expressed in the cells within the pannus. Similar to RANK, strong RANKL protein expression was observed in the bone-lining cells in the regions where the pannus was infiltrating the medulla of the epiphyseal portion of the femur (Fig. 8b). In the CIA-BIS group, RANKL protein was expressed in the cells within the pannus and the bone-lining cells (Fig. 8c), but the expression intensities were lower than those in the CIA-P group. In the CIA-BIS10 group, RANKL protein expression in the medulla of the epiphyseal portion of the femur and in the pannus was lower than that in the CIA and CIA-BIS groups (Fig. 8d). In all groups, no RANKL protein expression was noted in the lining cells of the metaphyseal and diaphyseal portions of the bone.

OPG expression in the synovial membrane and the medulla

In all groups, the bone-lining cells and the cells within the pannus were intensely stained. This was considered to result from nonspecific reactions. For this reason, there was no difference among the groups.

Western blotting

RANK protein

No expression was seen in the Cont group. Strong expression, slightly reduced expression, and no expression were seen in the CIA-P, CIA-BIS, and CIA-BIS10 groups, respectively (Fig. 9).

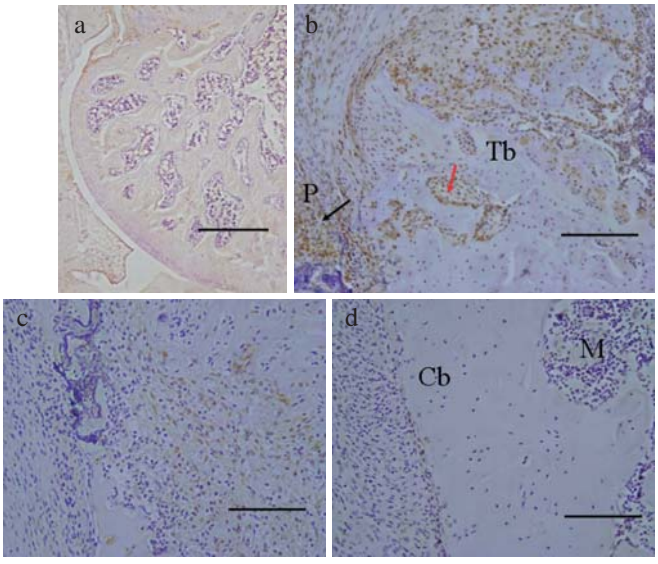

Fig. 7. Immunostained micrographs of the knee joints at 4 weeks after sensitization [receptor activator of nuclear factor κB (RANK) protein]. Sections of synovial–cartilage junctions from cont. (**a**), CIA-P (**b**), CIA-BIS (**c**), and CIA-BIS10 (**d**). *Cb* cortical bone, *Tb* trabecular bone, *M* bone marrow, and *P* pannus. **a** RANK was not expressed. **b** There was strong RANK protein expression in the pannus (*black arrow*) and also in the bone-lining cells near the pannus (*red arrow*). **c** RANK protein expression was found in the pannus and the bone-lining cells, but was weaker compared with that of the Cont group. **d** There was minor development of the pannus and little RANK protein expression was found. **a** *Bar* 500 μm; **b**, **c**, **d**: *bar* 100 μm

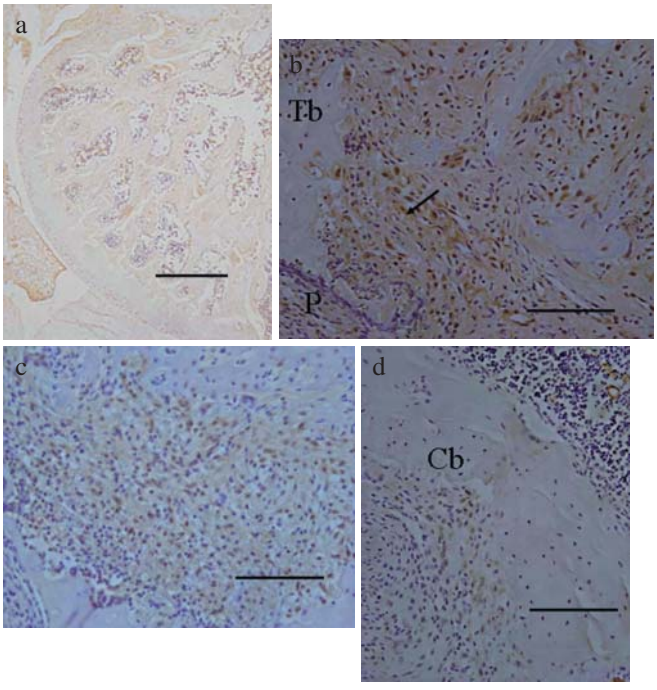

Fig. 8. Immunostained micrographs of the knee joints at 4 weeks after sensitization (RANKL protein). Sections of synovial–cartilage junctions from cont. (**a**), CIA-P (**b**), CIA-BIS (**c**), and CIA-BIS10 (**d**). *Cb* cortical bone, *Tb* trabecular bone, *M* bone marrow, *P* pannus. **a** RANKL was not expressed. **b** There was strong RANKL protein expression in the pannus (*black arrow*). **c** RANKL protein expression was found in the pannus and the bone-lining cells, but was weaker compared with that of the Cont group. **d** There was minor growth of the pannus, and little RANKL protein expression was found. **a** *Bar* 500 μm; **b**, **c**, **d** *bar* 100 μm

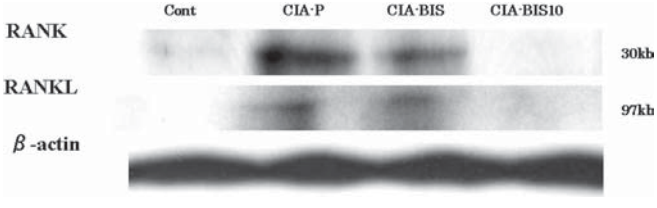

Fig. 9. Western blotting: rats killed at 4 weeks after the initial sensitization. The strong expression of both RANK and RANKL proteins was found in the CIA-P and CIA-BIS groups, and there was no difference between the two groups. No expression was found in the Cont and CIA-BIS10 groups

RANKL protein

No expression was seen in the Cont group. Similar expression was noted in the CIA-P and CIA-BIS groups, and no expression was seen in the CIA-BIS10 group (Fig. 9).

OPG protein

Expression of many proteins that react to anti-OPG antibody was seen in the CIA-P, CIA-BIS, and CIA-BIS10 groups. Evaluation was impossible because of the absence of specific OPG protein expression.

Discussion

The objective of this study was histological and immunohistological elucidation of the mechanisms of bone resorption by bisphosphonates in mature CIA rats. The advantage of using mature rats is that variations in the bone mass and the metabolic turnover in the bone during growth could be excluded owing to little variation in the BW and the bone mass in the Cont group throughout the experiment.[2] Furthermore, it has been reported that chronological changes of bone mass in mature CIA rats start from the early stages of arthritis with a decreased cancellous bone mass, and the pathology is similar to that of periarticular osteoporosis that complicates human RA.[2] In addition, it has been demonstrated that RANKL and RANK are expressed in the synovial cells and the bone-lining cells in CIA rats, and the RANKL–RANK system has been reported to play an important role in the activation of osteoclasts.[10,11]

Minodronic acid used in this study is a bisphosphonate with an estimated 1000-fold and 10–100-fold more potent inhibitory effect on bone resorption than etidronate and alendronate. According to a clinical trial randomized controlled trial (RCT) in Japan, the optimal dosage for osteoporosis treatment in humans is estimated to be 1 mg/day.[12] The dosage in this study (0.2 mg/kg/2 day) was approximately three- to fivefold greater than the optimal dosage for human osteoporosis, and was considered appropriate for experimental animals. There was a wide difference be-

tween the dosages of minodronic acid for the bone resorption and for inhibitory effects on calcification, and the inhibitory effects on bone resorption were considered as dosage dependent; thus, a tenfold amount (2.0 mg/kg/2 day) was also set.

The results in this study are summarized below. As previously reported,[10] the bone-lining cells and the cells within the pannus of CIA rats strongly expressed RANKL and RANK. In the minodronic acid-administered group, the dosage-dependent reduction of RANKL and RANK expression and TRAP-positive cells was confirmed by histology and Western blotting. However, with or without minodronic acid administration, no apoptotic osteoclasts were found in any groups.

The inhibitory mechanisms on bone resorption by bisphosphonates were considered to be derived from apoptosis induction in osteoclasts, and these agents have no effect on the differentiation process of osteoclasts.[13] In particular, there is a series of mechanisms in which bisphosphonates are rapidly deposited in the bone tissue because of their high affinity for calcium phosphate crystals in the bone,[14] and mevalotin metabolism in osteoclasts that incorporate them is inhibited with resultant apoptosis induction.[6] However, the results of this study did not support these mechanisms, and suggested the possibility that minodronic acid had effects on the differentiation of osteoclasts, by inhibiting the RANKL–RANK system. Halasy et al.[15] reported that alendronate and risedronate administration to osteoclasts derived from the rabbit femur reduced their resorption activities, but did not induce apoptosis in vitro. Pan et al.[16] reported that zoledronic acid stimulated OPG protein secretion on the cancellous bone-derived osteoblast-like cells obtained from human osteoarthritic patients, and suppressed RANKL protein expression. This report was consistent with the results of the present study in that RANKL protein expression was suppressed. RANKL is a membrane-spanning protein that belongs to the tumor necrosis factor (TNF) ligand family, induced by bone resorption factors such as active vitamin D3, PGE 2, parathyroid hormone, and IL-6 on feeder cells for osteoclast formation such as osteoblasts. Precursor cells of osteoclasts that express RANK, a RANKL receptor, receive a signal via binding to osteoblasts, and differentiate into osteoclasts. As a result of abnormal immune responses to self-antigen, induced inflammatory cytokines such as TNF-α, IL-1, and IL-6 are considered to stimulate the production of RANKL and cause differentiation and activation of osteoclasts.[17–25] The minodronic acid used in this study suppressed the expression of both RANKL and RANK, but it could not be determined whether this was caused by the direct suppression of RANKL and RANK or of either alone.

The presence of suppressive effects of bisphosphonates on arthritis has been reported.[26–31] Also in this study, arthritis was suppressed in the high-dosage minodronic acid-administered group (CIA-BIS10 group). Zwerina et al.[32] reported that administration of anti-TNF antibody (infliximab) and IL-1 antagonist (IL-1 receptor antagonist: IL-1Ra; anakinra) reduced the blood RANKL levels and the number of osteoclasts in rats with arthritis induced by TNF. Thus, the possibility of suppression of the production of inflammatory cytokines and then inhibiting the RANKL expression by minodronic acid is also considered.

This study revealed that minodronic acid had effects on the RANK–RANKL system and suppressed bone resorption, but their detailed action mechanisms cannot be elucidated because inflammatory cytokines such as TNF-α, IL-1, and IL-6 have not been evaluated. Active sites of minodronic acid could not be determined presumably because this was a protein-level study. In particular, it has also been reported that bisphosphonate administration enhances OPG expression;[33] thus, further studies are needed.

The results of this study on inhibitory mechanisms of bone resorption by minodronic acid using mature CIA rats demonstrated that minodronic acid inhibited the differentiation and activation of osteoclasts not by inducing apoptosis but by inhibiting the RANKL–RANK system, and thereby suppressing bone resorption.

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