Minodronic acid influences receptor activator of nuclear factor \( \kappa B \) ligand expression and suppresses bone resorption by osteoclasts in rats with collagen-induced arthritis

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 \( \kappa B \) (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-biotin nick 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.

Key words Bisphosphonate · Bone resorption · Collagen-induced arthritis (CIA) · Osteoclast · Receptor activator of nuclear factor \( \kappa B \) ligand (RANKL)

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.\(^5\) 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 0.5 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-\(\alpha\)]pyridine-3-yl)ethylidene]-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 \( (0 = \text{no arthritis}, 1 = \text{redness in at least one or more joints}, 2 = \text{inflammation in at least two or more joints or moderate inflammation in one or more joints}, 3 = \text{severe inflammation in one or more joints}, \text{and } 4 = \text{severest inflammation in one or more joints}) \) 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 sagitally 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 body, Streptavidin-biotin (SAB) method]. The posteromedial femoral condyle was mainly observed.

TRAP staining

According to the method described by Suzuki et al.,\(^4\) 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), naphthol 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),