Antiinflammatory and Chondroprotective Effects of the Aminobisphosphonate Incadronate (YM175) in Adjuvant Induced Arthritis

ATSUSHI MATSUO, TOSHIHIDE SHUTO, GO HIRATA, HIDESHI SATOH, YOSHIHIRO MATSUMOTO, HONGPU ZHAO, and YUKIHIDE IWAMOTO

ABSTRACT. ***Objective.*** Incadronate is a third-generation bisphosphonate that suppresses bone resorption and is used to treat skeletal disorders and prevent bone loss in pathological conditions. We evaluated its therapeutic potential and antiinflammatory effects in established adjuvant induced arthritis (AIA), a rat model of rheumatoid arthritis (RA).

Methods. Rats were administered incadronate subcutaneously at a dose of either 0.1 or 1.0 mg/kg/day, or 0.1 or 1.0 mg/kg/week, while a positive control group received phosphate buffered saline alone from Day 14 (after the onset of arthritis) to Day 42. The destruction of bone and cartilage and the antiinflammatory effects of incadronate in rats with established AIA were assessed during treatment, with reference to the arthritis index, hind paw volume, and radiological and histological examinations. To establish whether incadronate affects the migration of inflammatory cells, a chemotaxis assay was carried out using macrophage-like RAW 264.7 cells.

Results. *In vivo*, incadronate suppressed the clinical manifestations of AIA in a dose-dependent manner. *In vitro*, the various concentrations of incadronate suppressed the migration of macrophages, but the viability and adhesion of these cells were not suppressed.

Conclusion. Incadronate not only inhibits bone destruction but also reduces cartilage degeneration and joint inflammation in rats with established AIA. The mechanism underlying these antiinflammatory actions of incadronate may be attributable to the inhibition of macrophage migration to the site of inflammation. Bisphosphonates might be effective in preventing the progressive joint destruction and inflammation seen in patients with RA. (J Rheumatol 2003;30:1280–90)

Key Indexing Terms:
BISPHOSPHONATES INCADRONATE (YM175) BONE DESTRUCTION
CARTILAGE DESTRUCTION INFLAMMATION ADJUVANT INDUCED ARTHRITIS

Rheumatoid arthritis (RA) is characterized by chronic inflammation of the synovium, progressive erosion of the articular cartilage through pannus formation, and joint destruction. However, the etiology and pathogenesis of RA are not clearly understood. The degree to which bone involvement in RA can be controlled is also unsatisfactory, as the disease modifying antirheumatic drugs currently available have little overall effect on the course of bone destruction. Thus agents capable of suppressing both bone resorption and disease activity would be ideal candidates for the management of RA.

Bisphosphonates are synthetic analogs of pyrophosphate that contain nonhydrolyzable P-C-P bonds, have a high affinity for bone minerals, and inhibit bone resorption by osteoclasts[1]. These compounds have become the drugs of choice for the treatment of skeletal disorders involving excessive osteoclast mediated bone resorption, such as Paget's disease[2,3], tumor induced osteolysis and hypercalcemia[4,5], postmenopausal osteoporosis[6], and juxtaarticular and generalized osteoporosis in RA[7,8]. Since osteoclast mediated bone resorption is also known to be involved in the progressive bone destruction seen in RA, bisphosphonates may also be effective in preventing such bone destruction.

It has been reported that some bisphosphonates inhibit bone destruction in experimental animal models of RA, such as adjuvant induced arthritis (AIA) in rats[9-11]. We reported that a new generation aminobisphosphonate, incadronate, inhibited joint inflammation, as well as bone destruction, in rats with AIA when given before the onset of arthritis[12]. We investigated whether incadronate can inhibit bone and cartilage destruction and inflammatory indicators even when given after the onset of arthritis. We also noted that body weight tended to decrease when a high dose of incadronate was administered on a daily basis. Thus, new administration schedules aimed at preventing side effects and toxicity needed to be developed. A chondroprotective effect of zole-

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dronate has been described in experimental animals[13], but we were unable to clarify whether incadronate also had a chondroprotective effect. An antirheumatic and antiinflammatory effect of bisphosphonate has been described[7,8,14,15]. The mechanisms by which these compounds exert their antiinflammatory effects are not yet completely clear.

The aims of this study were thus: (1) to investigate whether incadronate can inhibit bone destruction and inflammatory indicators when used as a therapy for established AIA, (2) to compare the effectiveness of daily and weekly administration of incadronate in treating established AIA, (3) to investigate whether incadronate has a chondroprotective effect, and (4) to clarify the mechanisms by which these compounds exert their antiinflammatory effects.

MATERIALS AND METHODS

Animals. Female inbred Lewis rats were obtained from Charles River Japan (Kanagawa, Japan). The animals were housed in cages of 6 under a 12 h light/dark cycle and allowed food and water *ad libitum*; animals were kept according to the guidelines of the Ethics Committee for Animal Experiments at the Graduate School of Medical Sciences, Kyushu University, for one week prior to their first inoculation of adjuvant.

A total of 48 female Lewis rats, aged 8 weeks and weighing 150–160 g, were randomly allocated to 6 groups of 8 animals each. One of the groups was not subjected to the induction of arthritis or subsequent treatment, and acted as a normal control group. In the remaining groups, arthritis was induced in both hind paws of each rat. Freund's complete adjuvant was prepared by suspending heat killed *Mycobacterium butyricum* (lot 104320JA; Difco Laboratories, Detroit, MI, USA) in mineral oil (Nacalai Tesque, Kyoto, Japan) at a concentration of 10 mg/ml. A 0.1 ml aliquot was injected intradermally, once, into the proximal quarter of the tail on the first day of the experiment (Day 0), following a described method[12]. Incadronate (YM175; disodium dihydrogen cycloheptylamino-methylene bisphosphonate monohydrate) was kindly provided by Yamanouchi Pharmaceutical (Tokyo, Japan) and dissolved in phosphate buffered saline (PBS). The 4 treatment groups received incadronate at a dose of 0.1 or 1.0 mg/kg/day or 0.1 or 1.0 mg/kg/week, while a positive control group received PBS alone (Table 1). Incadronate at a dose of 1 mg/kg/day is 10 times higher than clinical doses for human treatment. Incadronate at a dose of 0.1 mg/kg/day is about the same as the clinical dose. Treatments were injected subcutaneously from Day 14 after the onset of arthritis to Day 42 (the end of the experiment), and clinical measures were assessed on Days 0, 15, 20, 25, 30, 35, and 42 after injection of adjuvant. On Day 42, all the animals were sacrificed and their hind limbs were collected for further examination.

Clinical measurements. Clinical measures, including measurements of body weight, an arthritis index, and hind paw volume, were assessed on the days detailed above. An arthritis index was assigned to every joint by a single observer, who remained blind to the animal's treatment group, using a standard method, as described by Kinne, *et al*[15]. Hind paw volume was measured using a volumetric apparatus (MK-550; Muromachi-kikai, Tokyo, Japan) as described[12].

Radiological examination. Radiographs were taken using an x-ray apparatus (C-SM; Softex, Tokyo, Japan) and industrial x-ray film (Fuji Photo Film, Tokyo, Japan). The x-ray apparatus was operated at 3 mV, with a 30 kV peak, 10 s exposure time, and a 45 cm tube-to-film distance for lateral projection. The severity of bone and joint destruction was assessed using a radiological index, as described by Barbier, *et al*[9].

Histological examination. On Day 42, 24 h after the final injection of incadronate or PBS, the animals were anesthetized by intraperitoneal injection of pentobarbital (Nembutal; Dainabot, Osaka, Japan) and fixed by intracardiac perfusion with 4% paraformaldehyde solution (pH 7.4). After perfusion, the hind limbs were removed from the distal femurs and radiographs were taken as described above. The hind paw specimens were then reimmersed in the same fixative at 4°C for 24 h, after which they were rinsed several times in PBS and decalcified in 10% EDTA solution (pH 7.2) at room temperature for 2 weeks. The ankle joints were then embedded in paraffin and sagittal sections 5 µm thick were prepared for histological examination. The sections were stained with safranin-O, and for tartrate resistant acid phosphate (TRAP) using a leukocyte acid phosphate kit (Sigma Chemical Co., St. Louis, MO, USA) at 37°C for 20 min in a moist chamber, before counterstaining with hematoxylin.

Qualitative assessment of articular cartilage degradation. Grading of the articular cartilage change in the tibiotarsal region was done by 2 observers who were blinded to the experimental outcome. The grading scheme used was described by Podworny, *et al*[13]. Briefly, the structural integrity of the tissue, the presence or absence of chondrocyte cloning, the degree of safranin-O staining, and tidemark integrity were assessed. The grade was a result of the sum of the scores, with maximum score of 14 indicating the most severe change.

Immunohistochemistry. The 5 µm thick sagittal sections were treated immunohistologically with ED1, a mouse anti-rat macrophage antibody (Serotec, Oxford, UK) to allow identification of the macrophages. The sections were deparaffinized and rehydrated through a graded series of ethanol. Endogenous peroxidase was then quenched by treating the sections with 3% hydrogen peroxide for 30 min. Nonspecific staining was blocked by incubation with normal rabbit serum (Nichirei Histofine SAB-PO) for 10 min at room temperature. The sections were then incubated with ED1 antibody at a dilution of 1:200 with 1% bovine serum albumin-PBS for 1.5 h at room temperature. The sections were incubated with secondary anti-mouse IgG, and then conjugated with biotin for 10 min and avidin-peroxidase complex for 5 min at room temperature. The immunoreaction was visualized with 3,3-diaminobenzidine as a substrate. The number of ED1 positive cells was calculated in 3 sections from 3 different specimens from each group. Three different visual fields were selected randomly from the proliferative synovial tissues of each section. We determined the number of ED1 positive cells in 9 different visual fields with the aid of a microscope (AX70TRF, Olympus Optical, Tokyo, Japan), using ×200 magnification.

Cell preparation. The mouse macrophage-like cell line RAW 264.7 was obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were cultured in α-minimum essential medium (α-MEM; Gibco, Gaithersburg, MD, USA) supplemented with 10% fetal bone serum (Gibco), 100 IU/ml penicillin, and 100 µg/ml streptomycin in a 95% air/5% CO_2 atmosphere at 37°C.

Chemotaxis assay. A chemotaxis assay was performed as described[16-18]. Briefly, polycarbonate filters, pore size 8 µm (Nuclepore, Pleasanton, CA, USA), were coated with 10 µl of 5% type IV collagen basement membrane, before being placed in Boyden chambers (Neuroprobe). The lower chambers were filled with α-MEM containing macrophage chemoattractant protein (MCP)-1 (10 µg/ml). Then, 6×10^5 viable cells suspended in serum-free α-MEM were placed in the upper compartments of the chamber with or without incadronate. After incubation for 5 h at 37°C in an atmosphere of 95% air/5% CO_2, the filters were removed, fixed with methanol, and stained with hematoxylin and eosin. The cells that had migrated to the lower surface of the filters were counted in 5 randomly selected microscope fields per filter (×200). All experiments were performed in triplicate.

Cell viability assay. The CellTiter-Glo™ luminescent cell viability assay was performed as described[19]. Briefly, this assay is a homogeneous method of determining the number of viable cells in a culture based on quantitation of the ATP present, which signals the presence of metabolically active cells. The RAW cell seeds were placed in a 96 well plate (1×10^4 cells/well), and incubated in serum-free α-MEM with or without various concentrations of incadronate (10^{-4} to 10^{-12} M) for 5 h. After incubation for 5 h at 37°C in an atmosphere of 95% air/5% CO_2, luminescence was recorded following the manufacturer's protocol.

RESULTS

On Day 42, the body weight indices for the incadronate 0.1 and 1.0 mg/kg/day groups were reduced by 0.1% and 0.4% compared with the PBS treated group, while those for the 0.1 and 1.0 mg/kg/week groups were increased by 0.5% and 0.2%, respectively. A significant decrease was thus not observed.

Administration of 1.0 mg/kg/day incadronate to rats with AIA resulted in a significant decrease in disease severity, as evaluated by the arthritis index and hind paw volume (Figures 1A and 2A). Weekly administration of a high dose of incadronate (1.0 mg/kg/week) had a similar effect to daily administration of a low drug dose (0.1 mg/kg/day) with respect to the arthritis index and hind paw volume (Figures 1B and 2B). On all the doses examined, the hind paw volume of the incadronate treated animals exhibited a significant dose-dependent reduction compared with the PBS treated group. On Day 42, the hind paw volumes of the 0.1 and 1.0 mg/kg/day incadronate groups had decreased to 65.2% and 61.4% of the initial value, respectively (Figure 2B). Incadronate at a dose of 0.1 or 1.0 mg/kg/day suppressed hind paw volume the same as dexamethasone at a dose of 0.25 mg/kg/day (data not shown). Dexamethasone at a dose of 0.25 mg/kg corresponds to methylprednisolone at a dose of 1.45 mg/kg.

Compared with the radiographs for the normal control rats, the PBS treated group exhibited severe bone destruction, with active osteophytosis. This was characterized by very thin trabeculae extending from the bone to the connective tissue. There were no visible joint spaces, and some bone had formed within the connective tissue. Incadronate suppressed these pathological changes in a dose-dependent manner (Figure 3C, 3D). On Day 14, radiographically, obvious bone destruction was not observed (Figure 3E). However, histologically, mild bone resorption and pannus formation were observed in the ankle joint. The hind limb radiological indices paralleled the clinical measures and were dose-dependent (Figure 4). The radiological index for each of the treatment groups was significantly lower than for the positive control group, with the high dose daily incadronate (1.0 mg/kg/day) group displaying the lowest degree of joint destruction. On Day 42, the radiological indices for the incadronate 0.1 and 1.0 mg/kg/day groups were reduced to 59.7% and 38.7%, respectively, compared with the PBS treated group, while indices for the 0.1 and 1.0 mg/kg/week dose groups were reduced to 87.1% and 61.3%, respectively (Figure 4).

The histological findings in the tibiotarsal region for each group on Day 42 are shown in Figure 5. In the PBS treated

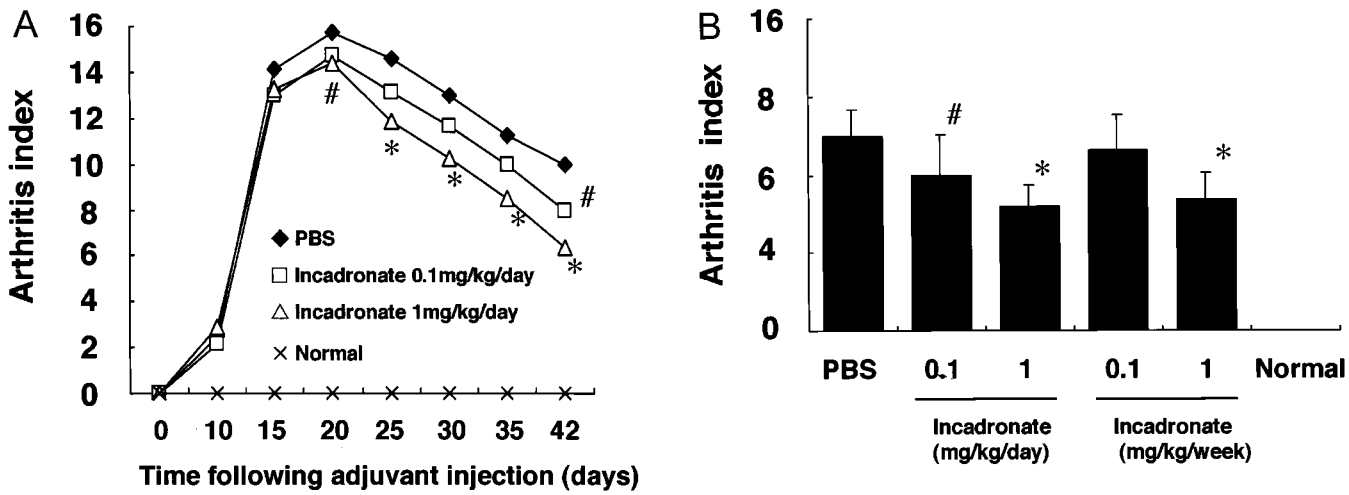

Figure 1. A. Time course of changes in the arthritis index. Incadronate (0.1 or 1 mg/kg/day) or PBS was injected subcutaneously every day from Day 14 after the onset of arthritis to Day 42. B. Effect of incadronate administration on the arthritis index on Day 42. Values are expressed as mean ± SEM (number of rats per group = 8). *$p < 0.01$, #$p < 0.05$ compared with the PBS treated positive control group (Mann-Whitney U-test).

Table 1. Experiment design.

Group	No. of Animals	Adjuvant Injection	Compound	Dose, mg/kg	Administration
A	8	+	Incadronate	0.1	Daily
B	8	+	Incadronate	1.0	Daily
C	8	+	Incadronate	0.1	Weekly
D	8	+	Incadronate	1.0	Weekly
E	8	+	PBS		Daily
F	8	–			

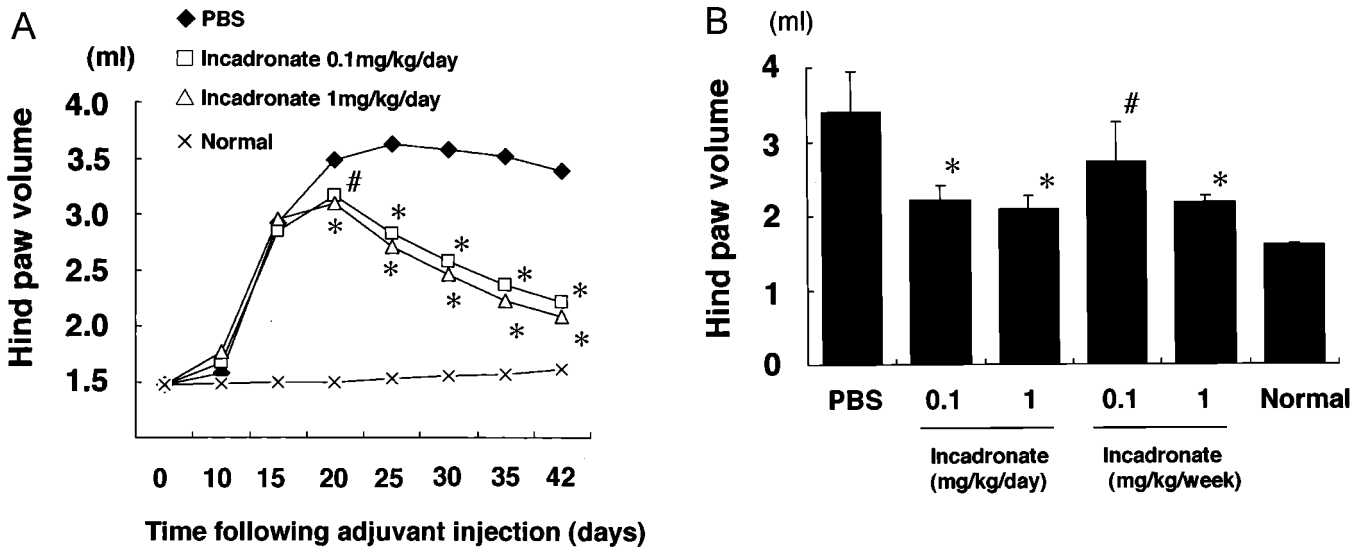

Figure 2. A. Time course of change in hind paw volume. Incadronate (0.1 or 1.0 mg/kg/day) or PBS was injected subcutaneously every day from Day 14 after the onset of arthritis to Day 42. B. Effect of incadronate administration on hind paw volume on Day 42. Values are expressed as mean ± SEM (number of rats per group = 8). *$p < 0.01$, #$p < 0.05$ compared with the PBS treated positive control group (Mann-Whitney U-test).

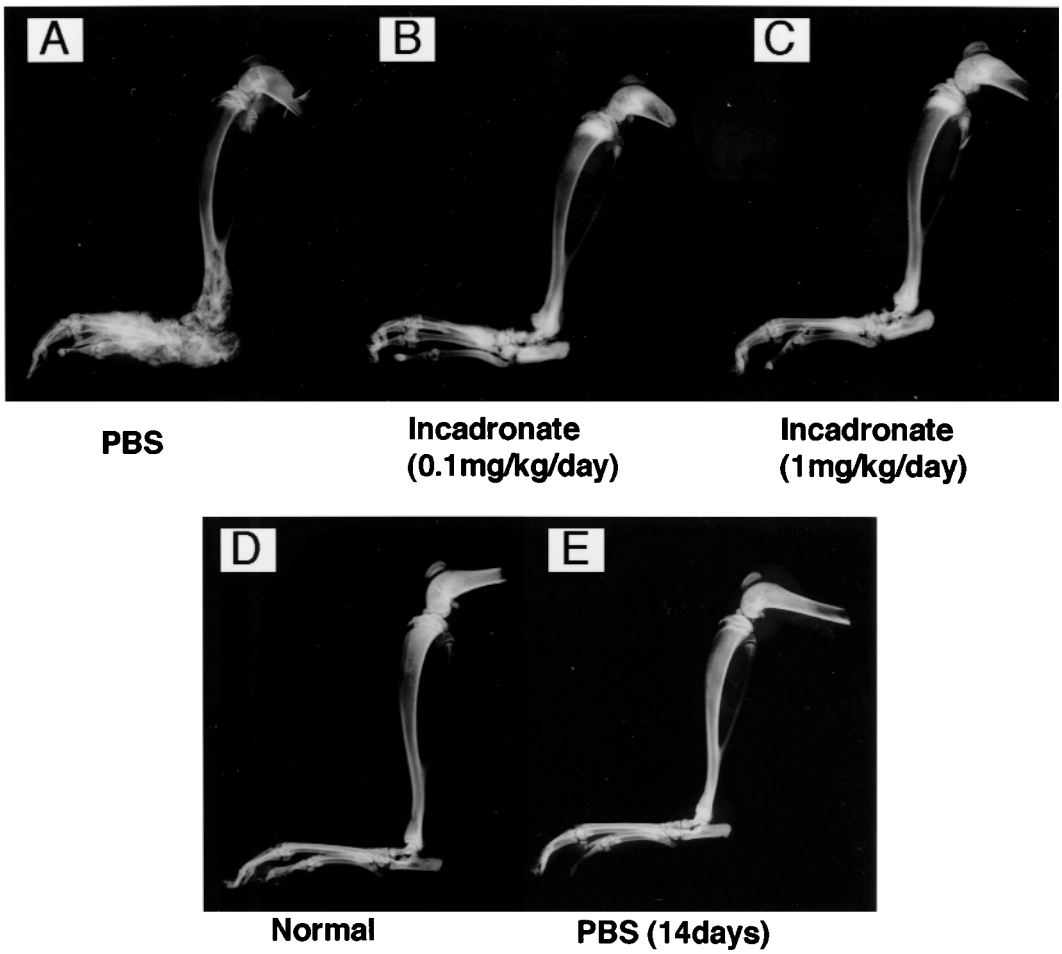

Figure 3. Radiographic appearance of rat hind limbs on Day 42. A. Normal control rats. B. PBS treated positive control rats. The hind paws show severe bone destruction with active osteophytosis, characterized by very thin trabeculae extending from the bone into the connective tissue. Significant aggravation of joint destruction was apparent in the positive control group during the late phase of the study compared with the normal control groups. C and D. Arthritic rats treated with 0.1 and 1.0 mg/kg/day incadronate, respectively. Early signs of active osteophytosis can be seen in the bone metaphysis, with reduced bone density and moderate alteration of the joint space. However, compared with the PBS treated group, the changes seen in the incadronate treated groups were mild or moderate. E. PBS treated positive control rats on Day 14 after onset of arthritis.

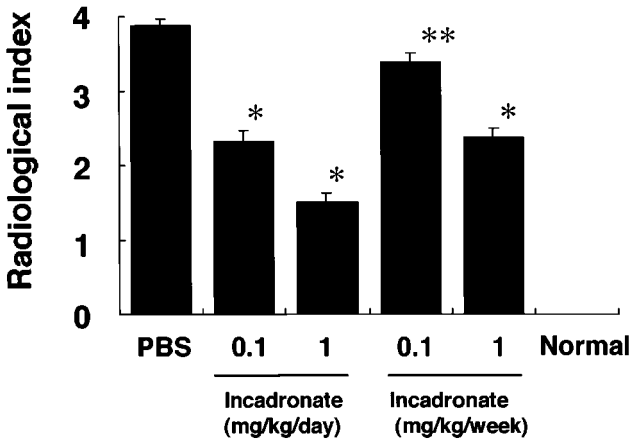

Figure 4. Radiological indices for the hind paws of the arthritic groups on Day 42. *p < 0.01, **p < 0.05 compared with the PBS treated positive control group (Mann-Whitney U-test). Values are expressed as mean ± SEM (number of rats per group = 8).

PBS treated group; however, the severity of changes was mild (Figures 5C and 6C). TRAP positive cells were occasionally seen near the bone surfaces, and the size of the TRAP positive cells in the marrow space was markedly reduced (Figure 6C). Incadronate suppressed these pathological changes and reduced the number of multinucleated TRAP positive cells in a dose-dependent manner, with the greatest reduction being observed in the 1.0 mg/kg/day incadronate group (Figure 6D). In this study in the incadronate treated group, TRAP positive multinuclear cells showed apoptotic changes, showing condensation and magnification of heterochromatins. These cells changed to round cells and detached from the surfaces of the bone trabeculae (Figure 7D).

The ankles of the positive control group showed variable degenerative changes in the cartilage, characterized by fibril formation and loss of proteoglycans. There was thinning of the subchondral bone and numerous breaks were observed in the osteochondral barrier (Figure 8B). Incadronate

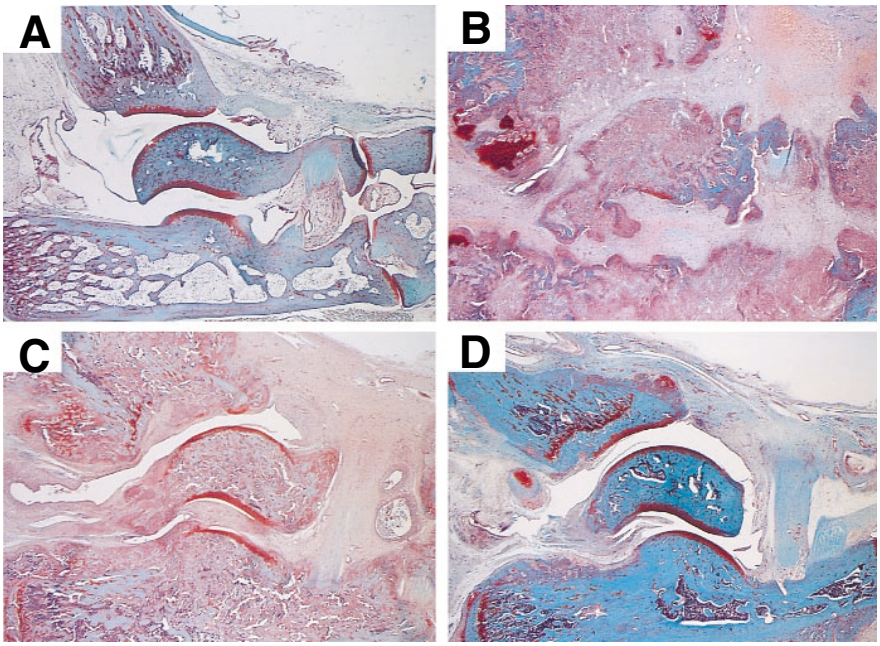

Figure 5. Histological appearance of the tibiotarsal regions on Day 42. Sections were stained with safranin-O. Original magnification ×40. A. Normal control rats. B. PBS treated positive control rats. The normal bone and joint structures have been completely destroyed. Marked cellular activity with intense osteolysis and osteogenesis is apparent. C and D. Arthritic rats treated with 0.1 and 1 mg/kg/day incadronate, respectively. The bone and joint structures are well preserved and little intense osteolysis can be observed.

group, the bone and joint were severely affected and the normal structure had been partially replaced by fibrous connective tissue. There was infiltration of numerous multinucleated TRAP-positive cells in the granulation tissue and bone marrow space, as well as on the surfaces of the bone trabeculae. The 0.1 mg/kg/day incadronate group showed destructive changes and cellular responses similar to the

partially prevented some of these degenerative changes and ameliorated the decrease in safranin-O staining of the articular cartilage in a dose-dependent manner (Figures 8C, 8D). It also suppressed pannus formation in the tibiotarsal regions in a dose-dependent manner. Thinning of the subchondral bone was not observed in the incadronate treated animals. Both PBS treated and incadronate treated

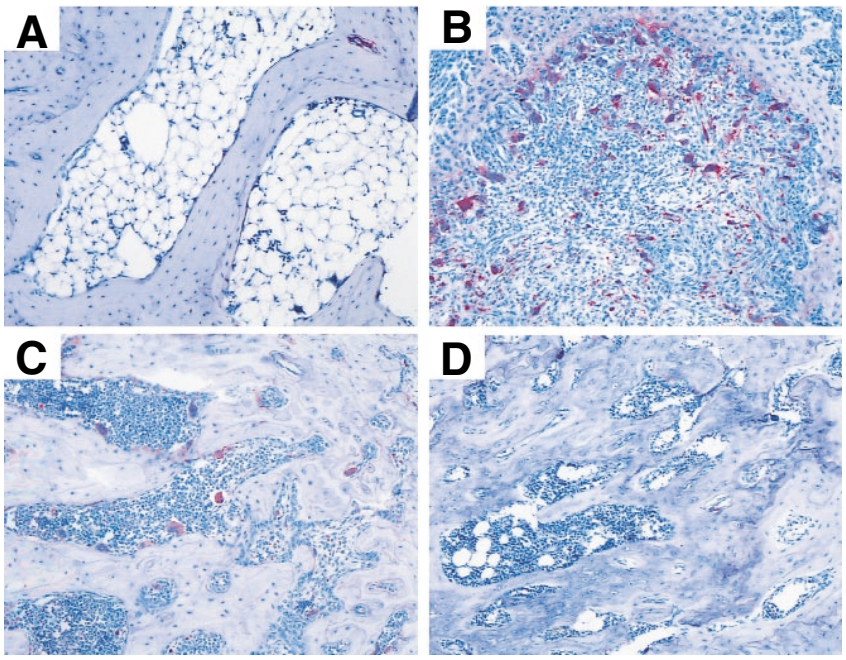

Figure 6. Magnified view of the calcaneus region on Day 42. Sections were stained for TRAP before counterstaining with hematoxylin. Original magnification ×100. A. Normal control rats. B. PBS treated positive control rats. There is marked infiltration of inflammatory cells and TRAP positive multinuclear cells in the connective tissue and bone marrow. C. Arthritic rats treated with 0.1 mg/kg/day incadronate. The number of TRAP positive multinuclear cells is markedly decreased. D. Arthritic rats treated with 1.0 mg/kg/day incadronate. TRAP positive multinuclear cells are seen rarely. The structure of bone trabeculae seems to be preserved.

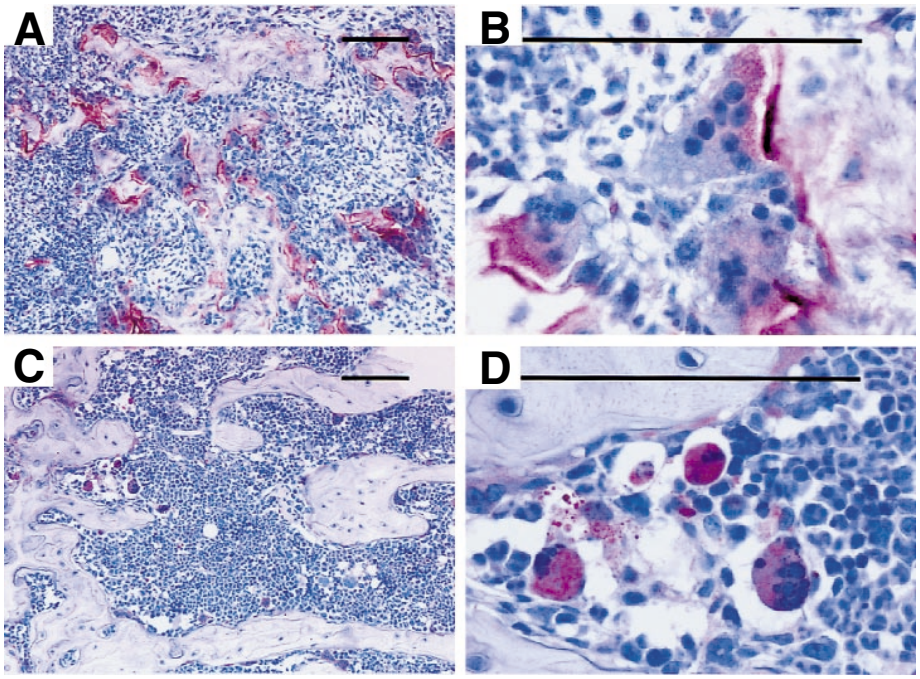

Figure 7. High power magnification of the calcaneus region on Day 42. Sections were stained for TRAP before counterstaining with hematoxylin. Scale bar = 50 µm. A. PBS treated positive control rats. Original magnification ×100. B. TRAP positive multinuclear cells are adjacent to the trabecular bone. Original magnification ×400. C. Arthritic rats treated with 0.1 mg/kg/day incadronate. Original magnification ×100. D. TRAP positive multinuclear cells showed apoptotic changes, with condensation and magnification of heterochromatins. These cells changed to round cells and detached from trabecular bone. Original magnification ×400.

groups showed degradation of articular cartilage compared to the normal groups. This is reflected in the grading score value. Although degradation was still evident in the incadronate treated animals, it was not as extensive as in the PBS treated group. Incadronate treatment at a dose of 1 mg/kg/day resulted in a 75.4% reduction in the severity of cartilage degradation, as reflected in the qualitative grading score, compared to the PBS treated groups (Figure 9). This suggests a partial chondroprotective effect by incadronate treatment.

In the ankles of the positive control group, pannus formation and infiltration of macrophages in the granulation tissue and bone marrow space were observed. Incadronate appeared to suppress this synovial tissue formation in a dose-dependent manner. Incadronate decreased the number of ED1 positive cells infiltrating the synovial tissue, also dose-dependently (Figures 10C, 10D, and 11).

To examine whether treatment of the cells with incadronate would affect the chemotaxis of the macrophages in response to MCP-1, a chemotaxis assay was carried out. The chemotaxis assay revealed that incadronate suppressed the migration of these cells in a dose-dependent manner. As can be seen in Figure 12, treatment with a high dose (10^{-4} M) and a low dose (10^{-8} M) of incadronate inhibited the chemotaxis of the cells to 45.8% and 64.2% of the control value, respectively. These results suggest that incadronate has an inhibitory effect on the migration of these cells.

To examine whether treatment of the cells with incadronate would affect cell viability, a cell viability assay was carried out. Statistically, a significant decrease in the cell number was not observed in the incadronate treated groups compared with nontreated groups. Therefore, a decrease of cell number on the lower surface of the filters was not cytotoxically effected by incadronate.

DISCUSSION

In our study, incadronate inhibited not only bone destruction but also joint inflammation and cartilage destruction in rats with established AIA; these results were confirmed both histologically and radiographically.

Further, weekly administration of high dose incadronate inhibited bone and joint destruction as effectively as daily administration of a low dose of the drug. These findings concur with the results of a study in which intermittent administration of incadronate was shown to be effective in ameliorating tumor related hypercalcemia[5]. No loss of body weight was observed in the rats receiving weekly incadronate, although we noted that body weight tended to decrease when a high daily dose of incadronate was given to AIA rats[12]. These results suggest that weekly administration of incadronate protects the bones and joints with no side effects or toxicity. Weekly administration of incadronate may therefore be an appropriate way of achieving bone and joint protection with fewer adverse effects in patients with RA.

Regarding the adverse effects of bisphosphonates on bone, administration of high doses of alendronate and pamidronate has been shown to inhibit bone turnover and cause cumulative microdamage *in vivo*[20,21]. Although no microdamage accumulation was observed in the bisphosphonate treated rats during our study, this possibility will need to be addressed in longterm studies with histological and radiographic monitoring to confirm the safety of longterm bisphosphonate use for human patients.

Administration of incadronate to rats with AIA resulted in a significant reduction of disease severity, as shown by the arthritis index and changes in hind paw volume. Antirheumatic and antiinflammatory effects have also been described recently for other bisphosphonates. A single infusion of pamidronate decreased the Ritchie Arthritis Index and the number of swollen joints in patients with active RA[7], while longterm administration of alendronate produced a significant reduction in interleukin 1 (IL-1), IL–6, and tumor necrosis factor-α (TNF-α) in patients with early RA[8]. Clodronate and pamidronate appear to inhibit the secretion of the proinflammatory cytokines IL-1ß, IL–6, and TNF-α from macrophage-like cells[22]. Thus, bisphosphonates may exert their antiarthritic effects by inhibiting the secretion of proinflammatory cytokines from macrophages and/or monocytes.

Pamidronate, alendronate, ibandronate, and risedronate have been shown to induce apoptosis in mouse macrophage-like cells *in vitro*[23,24]. We clarified that incadronate did not induce apoptosis of these cells in synovium *in vivo* (data not shown).

On the other hand, some bisphosphonates have cytotoxic and migration inhibitory effects on macrophages[25]. We also observed that incadronate inhibited the migration of these cells and pannus formation to joint spaces and bone marrow *in vivo* and the macrophage migration induced by MCP-1, but not cell viability and adhesion of macrophage cells *in vitro*. In addition, a chemotaxis assay was performed using human peripheral blood mononuclear cells. Incadronate also suppressed the migration of these cells in a dose-dependent manner (data not shown). *In vivo*, the sections were stained with TUNEL staining; however, macrophages of apoptosis were not observed in the synovium of AIA rats (data not shown). A high dose treatment of incadronate did not induce macrophage cell death. These results may suggest a novel mechanism underlying the antiinflammatory effects of bisphosphonates.

Although these compounds are well tolerated when administered intravenously, pamidronate, an aminobisphosphonate, is known to induce transient pyrexia in 10–50% of treated patients, occurring within 24–72 h and observed almost exclusively after the first treatment[26,27]. Such transient pyrexia has been described with other nitrogen-

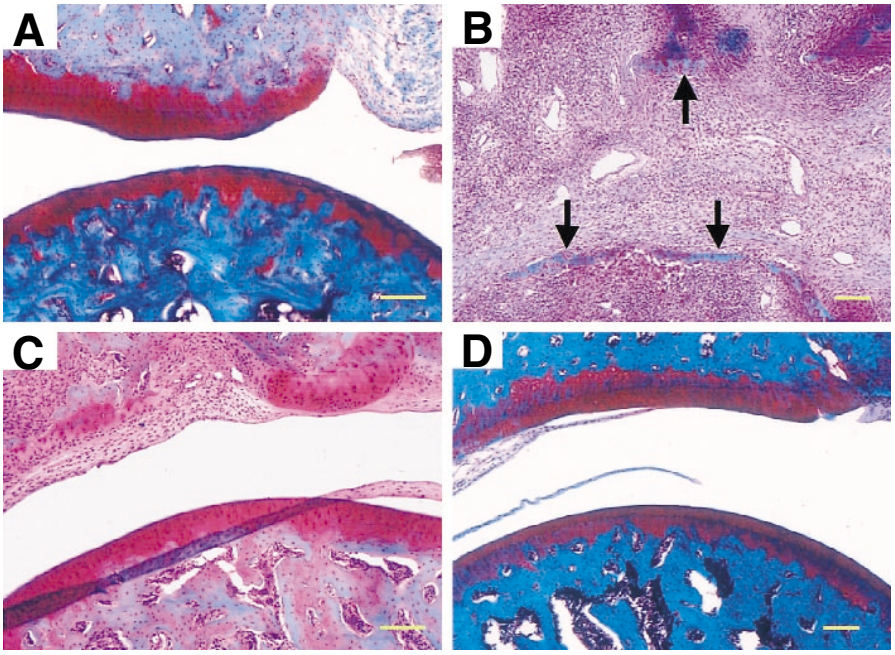

Figure 8. Histological appearances of tibiotarsal regions on Day 42. Sections were stained with safranin-O. Scale bar = 500 µm. A. Normal control rats. B. PBS treated positive control rats. Multiple layers of synovial cells occupy the tibiotarsal region. Loss of safranin-O staining is evident in the articular cartilage. C and D. Arthritic rats treated with 0.1 and 1.0 mg/kg/day incadronate, respectively. Preservation of safranin-O staining of articular cartilage is observed, and accumulation of multilayered synovial tissue has been reduced in a dose-dependent manner.

containing bisphosphonates[28,29], but rarely with clodronate[28,30], a non-aminobisphosphonate, raising the question of a role for the aminoalkane group of pamidronate in this inflammatory response[30,31]. On the other hand, patients treated with pamidronate displayed a reduction in joint swelling, and reported a decrease in morning stiffness and pain and an increased range of joint movement[7]. Longterm administration of alendronate produced a significant reduction in IL-1, IL–6, and TNF-α in patients with early RA[8]. Incadronate, a nitrogen-containing bisphosphonate, suppressed the inflammatory response in rat AIA in that study[12]. Early studies with free etidronate, clodronate, and pamidronate revealed a marked inhibition of pannus formation, inflammatory erosion of cartilage, and the pathological bone formation associated with rat AIA[10,32]. The antiinflammatory effects of clodronate have also been reported in murine AIA[33] and collagen induced arthritis in rats[14]. Therefore, we cannot clearly address the conflicting effects of the different classes of bisphosphonates on inflammation. A discrepancy in the antiinflammatory effects of these bisphosphonates may reflect differences in the structure of the agents, or the methods and terms of administration.

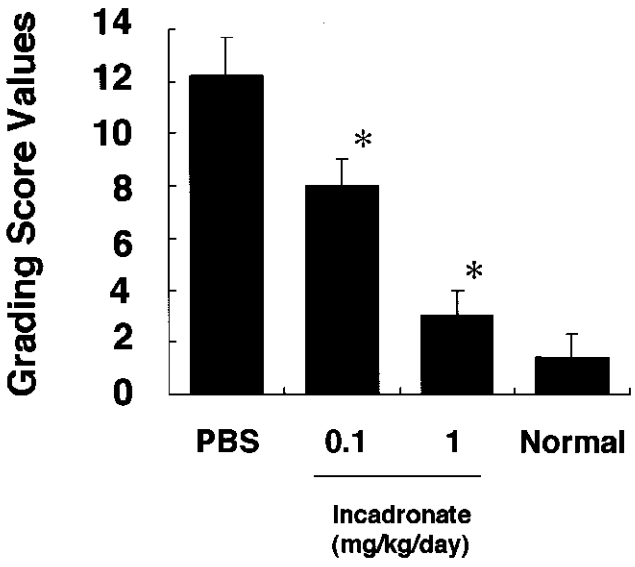

Figure 9. Qualitative grading score of the tibiotarsal regions on Day 42. *p < 0.01 compared with the PBS treated positive control group (Mann-Whitney U-test). Values are expressed as mean ± SEM (number of rats per group = 8).

We speculate that the aminobisphosphonate suppresses macrophage migration by inhibiting enzymes of the biosynthetic mevalonate pathway, preventing the production of isoprenoid compounds such as farnesyl pyrophosphate and geranylgeranyl pyrophosphate. The latter are required for the posttranslational prenylation and correct function of GTP-binding proteins such as Ras and Rho[34-36], known to play a key role in the regulation of cell structure or cell migration. It is likely that incadronate induces deformity of

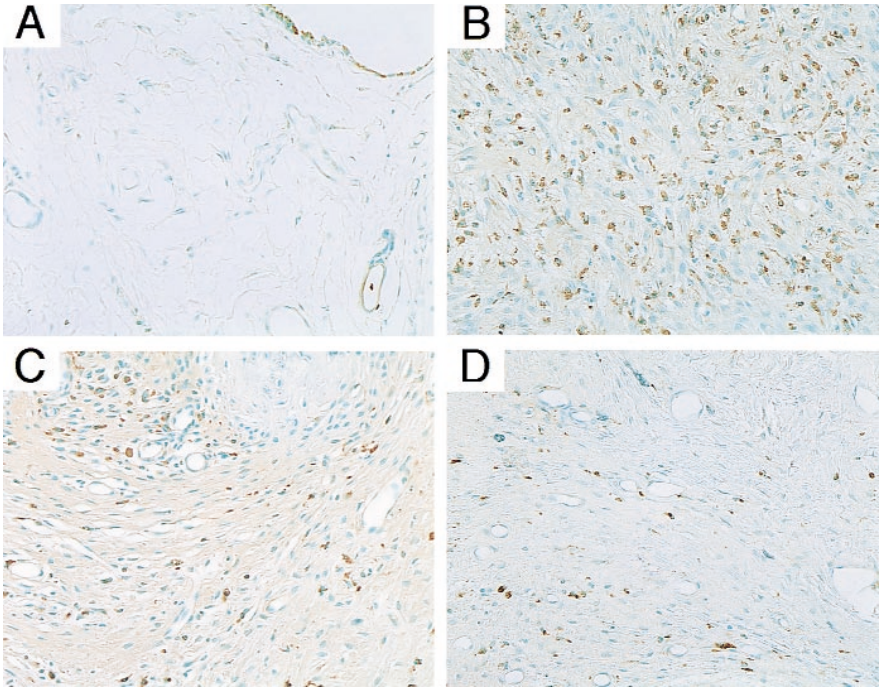

Figure 10. High power magnification of the synovial tissue region on Day 42. Sections were immunostained for ED1 before counterstaining with methyl green. Original magnification ×200. A. Normal control rats. B. PBS treated positive control rats. Marked infiltration of inflammatory cells in the connective tissue can be seen. C. Arthritic rats treated with 0.1 mg/kg/day incadronate. The number of ED1 positive cells is markedly reduced. D. Arthritic rats treated with 1.0 mg/kg/day incadronate. ED1 positive cells are seen rarely.

cell structure in RAW264 cells as a consequence of loss of prenylation and function of proteins such as Ras or Rho. Incadronate may also inhibit macrophage migration by inhibiting prenylation since statin drugs (HMG-CoA reductase inhibitors), known inhibitors of the mevalonate pathway, can also cause an antiinflammatory effect[37,38].

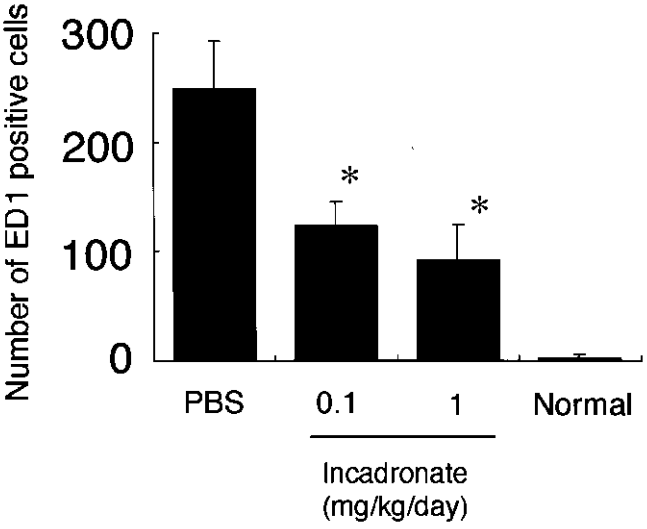

Figure 11. Number of ED1 positive cells/section from each group on Day 42. *p < 0.01 compared with the PBS treated control group (Mann-Whitney U-test). Values are expressed as mean ± SEM (n = 8).

Incadronate treatment appeared to prevent some of the degenerative changes induced by adjuvant injection, such as loss of cartilaginous tissue and pannus formation. *In vitro* studies have shown that zoledronate inhibits IL-1ß induced

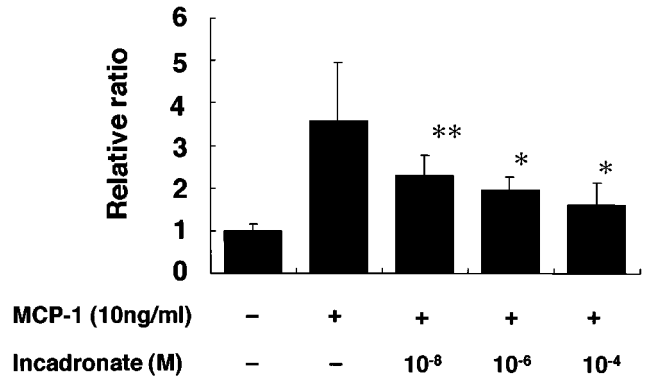

Figure 12. Chemotaxis of RAW 264.7 cells in response to MCP-1. Cells were incubated with various concentrations of incadronate that were added into the upper compartment of a modified Boyden chamber. The indicated concentration of MCP-1 (10 µg/ml) was added to the lower compartment and incubated 5 h at 37°C. The migration was measured as described in Materials and Methods. Cells migrated to the lower surface of the filters — 49 cells per filter in the control group. The macrophage migration of 10^{-8} M, 10^{-6} M, and 10^{-4} M incadronate had decreased to 64.2%, 55.4%, and 45.8% of the initial value, respectively. Values are expressed as mean ± SEM. *p < 0.01, **p < 0.05 compared with the control group (Mann-Whitney U-test).

matrix metalloprotease (MMP)-1 secretion by bone marrow stromal cells[39], while alendronate also inhibits MMP-13 secretion by synovial tissue[40]. A single injection of zoledronate also reduced urinary type-II collagen C-telopeptide degradation by MMP in patients with Paget's disease[41]. These chondroprotective effects seem to result from a direct effect of bisphosphonates on cartilage matrix degradation, which is achieved by inhibiting the activity or decreasing the production of MMP by synoviocytes or chondrocytes. As reported[22], some bisphosphonates can inhibit lipopolysaccharide induced IL-1ß, IL–6, and TNF-α secretion by macrophage-like cells[22]. These cytokines play an important role in the degeneration of articular cartilage as well as in joint inflammation and destruction. TNF-α stimulates the resorption and inhibits the synthesis of proteoglycans in cartilage[42], while human recombinant IL-1 and TNF-α have been shown to mediate the suppression of glycosaminoglycan synthesis in cultured rat costal chondrocytes[43,44]. There have been no reports of bisphosphonates acting directly on cartilage, suggesting that they prevent cartilage destruction indirectly by inhibiting the secretion of proinflammatory cytokines from macrophages and/or monocytes. Thus, bisphosphonates might inhibit cartilage destruction in patients with RA, although no evidence for a chondroprotective effect in humans is presently available. This possibility will need to be investigated in longterm studies in patients with RA by histological and radiographic monitoring of their joints.

Our results indicate that incadronate inhibits not only bone destruction but also cartilage degeneration and joint inflammation in established AIA. These findings suggest that bisphosphonates could prevent the bone loss and cartilage destruction associated with RA, and would be a logical therapeutic option.

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