Micro-CT imaging analysis for the effects of ibandronate and eldecalcitol on secondary osteoporosis and arthritis in adjuvant-induced arthritis rats

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ABSTRACT

We investigated the effects of ibandronate, a bisphosphonate; eldecalcitol, an active vitamin D3 analogue; and combination treatment with both agents on secondary osteoporosis and arthritis using rats with adjuvant-induced arthritis. Arthritis was induced in 8-week-old male Lewis rats. Rats were randomized into four treatment groups and an untreated normal control group: ibandronate, eldecalcitol, ibandronate + eldecalcitol, vehicle, and control. Paw thickness was measured to evaluate arthritis. Joint destruction was evaluated histomorphometrically by the ankle joint stained with Fast Green and safranin O. The femur and lumbar spine were scanned using dual-energy X-ray absorptiometry, and the distal femur was scanned using micro-computed tomography for bone mineral density (BMD) and trabecular microstructural evaluations. Ibandronate and/or eldecalcitol increased BMD in both the lumbar vertebrae and femur and improved several microstructural parameters (bone volume/total volume, structure model index, trabecular number, and trabecular separation of the distal femur). In addition, there was an additive effect of combination treatment compared with single treatments for most trabecular parameters, including BMD and bone volume. However, ibandronate and/or eldecalcitol did not inhibit arthritis and joint destruction. Combination treatment with ibandronate and eldecalcitol may be effective for secondary osteoporosis associated with arthritis.

Rheumatoid arthritis (RA) is a chronic and progressive autoimmune disease characterized by the presence of an inflammatory synovitis; it results in destruction of joint cartilage and bone (11). In RA, persistent inflammation leads to periarticular and generalized osteoporosis (16, 27), which increases the risk for fragility fractures compared with patients with primary osteoporosis (22). Because severe joint destruction and fragility fractures increase mortality rates (21, 29, 44), it is important to prevent destruction of joint cartilage, arthritis, and bone loss in patients with RA.

Tumor necrosis factor-α inhibitors (TNFi) and methotrexate are commonly used to suppress joint destruction in patients with RA. Several studies reported that TNFi and methotrexate improved the spine or hip bone mineral density (BMD) in the patients with RA (4, 8, 13), while Kim et al. reported that TNFi and methotrexate did not reduce the incidence of osteoporotic fractures (23). Moreover, Ochi et al. reported that despite improvements in disease activity and functional disability, incidence of fragility fractures did not decrease in patients with RA.
pared suspension of *Mycobacterium tuberculosis* H37Ra (Difco Laboratories, Detroit, MI, USA) in paraffin oil (5 mg/mL) into the footpad of the right hind paw (50 μL).

**Experimental protocol.** Normal untreated rats were used as a control group. From day 21 after adjuvant injection, the control and AIA rats were divided into five groups (*n* = 19–20, in each group): 1) control group, normal control rats for the AIA group; 2) vehicle group, AIA control rats treated with isotonic sodium chloride solution (Otsuka Pharmaceutical Factory, Tokushima, Japan) for vehicle of IBN and medium-chain triglyceride (The Nisshin Oilliio Group, Tokyo, Japan) for vehicle of ELD; 3) IBN group, AIA rats treated with IBN (subcutaneously, once every 2 weeks, 10 μg/kg) (Bonviva; Chugai Pharmaceutical Co., Ltd., Tokyo, Japan); 4) ELD group, AIA rats treated with ELD (orally, once daily, 30 ng/kg/day) (Chugai Pharmaceutical Co., Ltd.); and 5) IBN + ELD group, AIA rats treated with a combination of IBN + ELD (Fig. 1). The dose of IBN or ELD was based on previous studies (36, 39), in which IBN and ELD elevated rat femoral and lumbar spinal BMD. Rats were allowed ad libitum access to tap water and commercial standard rodent chow (CE-7; Clea Japan, Tokyo, Japan) and housed in a controlled environment (temperature 23 ± 2°C, humidity 40 ± 20%) with a 12-h light-dark cycle. After 2 or 4 weeks of treatment, rats were euthanized with an injection of sodium pentobarbital (150 mg/kg body weight, intraperitoneally) (Nembutal; Sumitomo Dainippon Pharma Co., Ltd., Osaka, Japan). The Animal Research Committee of our institute approved the protocol for all animal experiments, and all animal experiments adhered to the “Guidelines for Animal Experimentation” of the university.

In this study, we investigated the effects of IBN, ELD, and their combination on secondary osteoporosis and arthritis using adjuvant-induced arthritis (AIA) rats. The AIA rat is a widely used experimental animal model of RA because it shows similarities with human RA (33, 41).

**MATERIALS AND METHODS**

**Animals.** This study used 8-week-old male Lewis rats (Japan SLC, Shizuoka, Japan). Adjuvant arthritis was induced by subcutaneous injection of a pre-
Bisphosphonate and vitamin D

Tissue preparation. Left femurs and lumbar vertebrae (L2–L4) were harvested and used for measurement of BMD by dual-Xray absorptiometry (DXA); left femurs were used for analysis of trabecular bone microarchitecture by micro-computed tomography (micro-CT). Right hind ankles were also harvested for evaluation of joint destruction.

Clinical measurements of arthritis by paw thickness. Clinical signs of arthritis in each hind paw were assessed once weekly by investigators blind to the treatment group, as previously described (25, 42). Hind footpad width was also measured once weekly with calipers (Dial Thickness Gauge; Ozaki Mfg. Co., Ltd., Tokyo, Japan) (35).

Joint destruction of ankle joint. Right hind ankles were harvested for histologic examination. After removal of the surrounding soft tissue, the ankles were fixed in 10% formalin and decalcified in 10% ethylenediamine tetraacetic acid for 4 weeks. Following dehydration and paraffin embedding, 5-μm-thick sagittal sections were cut at the center of the ankle.

To assess the infiltration of inflammatory cells and bone damage, sections were stained with hematoxylin and eosin, and 0.1% Fast Green for 10 min and then 0.1% safranin O for 7 min. The severity of joint destruction was evaluated with two previously reported methods (7, 9, 35). Briefly, proteoglycan loss was classified into 4 scores: 0, no loss; 1, mild loss; 2, severe loss; 3, complete loss of staining for proteoglycan (7, 9). Second, cartilage destruction was classified into 6 scores: 0, normal; 1, minimal (loss of safranin O staining only); 2, mild (loss of safranin O staining and mild cartilage thinning and fissuring); 3, moderate (moderate cartilage destruction with one to three sites of minor-focal depth reaching the middle zone); 4, marked (marked cartilage destruction with more than three sites of minor-focal depth reaching the deep zone); and 5, severe (severe cartilage destruction with macro-focal cartilage destruction reaching the tidemark) (9, 35).

BMD measurement. The total length of the femur was trisected into three regions: proximal, middle, and distal. BMD of the proximal, middle, distal femur, and L2–L4 was measured using DXA (QDR-4500 Delphi; Hologic, Bedford, MA, USA).

Micro-computed tomography examination. Micro-CT was used to assess secondary osteoporosis. The excised left femurs from rats in the 5 groups that were treated for 4 weeks (n = 5 each) were secured in a sample holder. Micro-CT was performed with a micro-CT 35 instrument (SCANCO Medical, Zurich, Switzerland) with an isotropic voxel size of 10 μm, an integration time of 400 ms, energy of 70 kVp, and a current of 114 μA. Each measurement included 928 slices, corresponding to a 9.28-mm-thick cross section of the distal femur. The CT values for the lower and upper thresholds were 220 and 1000, respectively. The regions of interest of femoral trabecular bone were chosen for analysis using cross-sectional images from micro-CT scanning. Three hundred slices of distal femoral metaphysis starting at 1 mm from the end of the growth plate were analyzed. Bone volume/total volume (BV/TV), trabecular number (Tb.N), trabecular separation (Tb.Sp), trabecular thickness (Tb.Th), structure model index (SMI), connectivity density (Conn.D), and cortical thickness (Ct.Th) at the left distal femur were measured according to the guidelines of the American Society for Bone and Mineral Research (2).

Statistical analyses. The Statistical Package for the Biosciences software (SPBS) version 9.6 (Akita University, Akita, Japan) was used for statistical analyses (28). Continuous variables were expressed as mean ± standard deviation (SD). Differences between groups at each time point were evaluated using one-way analysis of variance (ANOVA). Multiple comparisons were made using Scheffe’s and Dunn’s post hoc tests, as appropriate. Nonparametric data, including proteoglycan loss score and cartilage destruction score, were analyzed by Dunn’s method. Parametric data were analyzed by Scheffe’s method. Data were analyzed by one-way ANOVA followed by a least significant difference test. Values of *P* < 0.05 were considered significant.

RESULTS

Arthritis and joint destruction

Fig. 2 shows representative cases of the hind footpad in each group. The paw thickness of rats in all AIA groups was significantly greater than that of rats in the control group. However, there were no significant differences in the paw thickness among the AIA groups.

Fig. 3 shows representative Fast Green-safranin O staining of ankle joints in each group at 4 weeks. Compared with the control group, severe loss of proteoglycan and severe cartilage destruction were seen in all AIA groups. There was no clear difference of proteoglycan loss score and cartilage de-
BMD
At the end of 2 and 4 weeks, femoral and lumbar BMD was significantly decreased in all AIA groups (vehicle, IBN, ELD, and IBN + ELD groups) con-

Fig. 2 Representative pictures of the left hind limb of rats in each group at 4 weeks. CON (a), vehicle (b), IBN (c), ELD (d), and IBN + ELD (e). There was no difference in paw thickness in the AIA groups (vehicle, IBN, ELD, and IBN + ELD). A white double arrow indicates the paw thickness. CON, control; IBN, ibandronate; ELD, eldecalcitol

Fig. 3 Representative pictures of Fast Green-safranin O staining of ankle joints. CON (a), vehicle (b), IBN (c), ELD (d), and IBN + ELD (e) at 4 weeks (magnification ×10). CON, control; IBN, ibandronate; ELD, eldecalcitol

struction score among the vehicle, IBN, ELD, and IBN + ELD groups (Fig. 4).
Bisphosphonate and vitamin D

At 2 weeks experiment, IBN monotherapy significantly increased BMD of distal femur and ELD monotherapy significantly increased BMD of proximal and distal femur, which consist of cancellous bone, compared with the vehicle group ($P < 0.05$). An additive increase was observed in distal femoral BMD of the IBN + ELD groups ($P < 0.05$). On the other hand, proximal femoral BMD in the ELD and IBN + ELD groups was significantly increased compared with the IBN group ($P < 0.05$). However, there were no significant differences in the middle femoral BMD among the AIA groups.

At 4 weeks, IBN and ELD monotherapy increased the proximal and distal femoral BMD compared with the vehicle group ($P < 0.05$). Furthermore, an additive increase was observed at the BMD of proximal and distal femur in the IBN + ELD groups, compared with IBN alone or ELD alone ($P < 0.05$). At the end of 4 weeks, BMD of the middle femur, which consists of cortical bone, was significantly increased in the ELD and IBN + ELD groups compared with the vehicle group ($P < 0.05$).

IBN and/or ELD treatment significantly increased lumbar BMD at 2 and 4 weeks compared with the vehicle ($P < 0.05$). In addition, IBN + ELD significantly increased lumbar BMD compared to IBN or ELD monotherapy ($P < 0.05$), and increased lumbar BMD at the same level as the control group at 4 weeks.

**Micro-CT**

Fig. 5 shows representative micro-CT images in each group. AIA induced severe trabecular bone loss and thinning of cortical bone at the distal femur at 4 weeks in the vehicle, IBN, ELD, and IBN + ELD groups compared with the control group. Treatment with IBN + ELD increased trabecular bone and cortical thickness (Fig. 5). AIA (vehicle, IBN, ELD, and IBN + ELD) groups had significant decreases in BV/TV, Conn D, Tb.Th, and Ct.Th as well as increases in SMI compared with the control group ($P < 0.05$). Tb.N of the vehicle, ELD, and IBN + ELD groups was significantly lower than that of the control group ($P < 0.05$). Tb.Sp in the vehicle group was significantly higher than that in the control group ($P < 0.05$). Tb.N of the vehicle, ELD, and IBN + ELD groups was significantly lower than that of the control group ($P < 0.05$). Tb.Sp in the vehicle group was significantly higher than that in the control group ($P < 0.05$). IBN or ELD monotherapy significantly increased Tb.N and decreased SMI and Tb.Sp compared with the vehicle group ($P < 0.05$). IBN + ELD significantly increased BV/TV and Tb.N, and decreased SMI and Tb.Sp compared with the vehicle group ($P < 0.05$) (Table 2 and Fig. 5). IBN and/or ELD treatment for 4 weeks did not differ from the vehicle group in terms of Ct.Th.
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volume at 4 weeks, whereas IBN and/or ELD did not lead to recovery of cortical BMD (middle femur) at 2 weeks. Similar negative findings were seen for cortical thickness, arthritis, and joint destruction in all AIA rats.

There are several reports on combination therapy with IBN + ELD. Sakai et al. reported that combination treatment with IBN + ELD acted synergistically to reduce bone resorption without suppressing bone formation in ovariectomized rats (36). Takada et al. reported that combined use of IBN + ELD for

DISCUSSION

In the present study, we demonstrated that IBN and/or ELD increased BMD of trabecular bone in both the lumbar vertebrae and femur and improved several microstructural parameters (BV/TV, SMI, Tb.N, and Tb.Sp) of the trabecular bone in the distal femur. There was an additive effect of combination treatment IBN + ELD compared with single treatments for most trabecular parameters, including cancellous BMD (proximal and distal femur) and bone volume at 4 weeks, whereas IBN and/or ELD did not lead to recovery of cortical BMD (middle femur) at 2 weeks. Similar negative findings were seen for cortical thickness, arthritis, and joint destruction in all AIA rats.

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Table 1 BMD (g/cm²) in each group at 2 and 4 weeks

|                | CON     | Vehicle | IBN     | ELD     | IBN + ELD |
|----------------|---------|---------|---------|---------|-----------|
|                |         |         |         |         |           |
| **Femur**      |         |         |         |         |           |
| **2 weeks**    |         |         |         |         |           |
| Proximal       | 0.242 ± 0.006 | 0.183 ± 0.012* | 0.195 ± 0.009* | 0.208 ± 0.014* | 0.210 ± 0.006* |
| Middle         | 0.210 ± 0.008 | 0.180 ± 0.012* | 0.183 ± 0.008* | 0.189 ± 0.011* | 0.187 ± 0.013* |
| Distal         | 0.235 ± 0.012 | 0.143 ± 0.020* | 0.169 ± 0.010* | 0.168 ± 0.010* | 0.190 ± 0.017* |
| **4 weeks**    |         |         |         |         |           |
| Proximal       | 0.261 ± 0.011 | 0.165 ± 0.007* | 0.194 ± 0.008* | 0.200 ± 0.010* | 0.213 ± 0.012* |
| Middle         | 0.228 ± 0.010 | 0.165 ± 0.013* | 0.176 ± 0.006* | 0.183 ± 0.013* | 0.182 ± 0.009* |
| Distal         | 0.247 ± 0.015 | 0.134 ± 0.015* | 0.166 ± 0.006* | 0.177 ± 0.027* | 0.205 ± 0.015* |
| **Lumbar**     |         |         |         |         |           |
| **2 weeks**    |         |         |         |         |           |
| Proximal       | 0.212 ± 0.005 | 0.171 ± 0.008* | 0.184 ± 0.007* | 0.193 ± 0.008* | 0.201 ± 0.009* |
| Middle         | 0.220 ± 0.006 | 0.168 ± 0.007* | 0.188 ± 0.010* | 0.198 ± 0.005* | 0.210 ± 0.011* |
| **4 weeks**    |         |         |         |         |           |
| Proximal       | 0.220 ± 0.006 | 0.168 ± 0.007* | 0.188 ± 0.010* | 0.198 ± 0.005* | 0.210 ± 0.011* |

n = 9–10 per group. Values represent mean ± SD.

BMD, bone mineral density; CON, control; IBN, ibandronate; ELD, eldecalcitol.

*P < 0.05 vs. CON by Scheffe’s method. †P < 0.05 vs. Vehicle by Scheffe’s method. ‡P < 0.05 vs. IBN by Scheffe’s method. ¶P < 0.05 vs. ELD by Scheffe’s method.

Fig. 5 Representative micro-CT pictures in the distal femur. CON (a), vehicle (b), IBN (c), ELD (d), and IBN + ELD (e). CON, control; IBN, ibandronate; ELD, eldecalcitol.
Bisphosphonate and vitamin D

Produced cytokine production by spleen cells in AIA rats (18). In contrast, Oelzner et al. reported that vitamin D did not affect inflammation and joint destruction in AIA rats (31).

There are several limitations to the present study. First, the model used in the previous study was collagen-induced arthritis (24, 37, 40, 43), which has been reported to produce less severe articular destruction than AIA (3). Second, the duration of ELD administration was short compared with previous experiments (24, 40). Due to severe joint destruction or short duration of ELD administration, the effect of the ELD treatment might have been difficult to evaluate. Third, the timing of treatment 3 weeks after the onset of arthritis might have been too late to evaluate the effect of treatments on suppressing joint destruction.

In conclusion, IBN and/or ELD treatment increased BMD in both the lumbar vertebrae and femur and improved some microstructural parameters (BV/TV, SMI, Tb.N, and Tb.Sp) of the distal femur in AIA rats. However, IBN and/or ELD treatment did not inhibit arthritis. These results suggest that combination treatment of IBN + ELD may be effective for secondary osteoporosis associated with RA, but another treatment might be necessary for arthritis associated with RA.

Table 2  Micro-CT parameters at 4 weeks

|             | CON   | Vehicle | IBN    | ELD    | IBN + ELD |
|-------------|-------|---------|--------|--------|-----------|
| BV/TV (%)   | 0.165 ± 0.031 | 0.004 ± 0.001* | 0.031 ± 0.006* | 0.030 ± 0.027* | 0.049 ± 0.029*† |
| Conn.D (1/mm²) | 70.13 ± 4.150 | 3.967 ± 0.213* | 3.175 ± 0.079*† | 3.140 ± 0.324*† | 2.867 ± 0.403*† |
| SMI       | 1.852 ± 0.221 | 0.41 ± 0.39* | 0.042 ± 0.009* | 0.044 ± 0.003* | 0.045 ± 0.006* |
| Tb.N (1/mm) | 2.534 ± 0.149 | 0.909 ± 0.153* | 1.230 ± 0.178* | 1.931 ± 0.542*† | 2.113 ± 0.348*† |
| Tb.Th (mm) | 0.081 ± 0.007 | 0.042 ± 0.009* | 0.434 ± 0.032* | 0.542 ± 0.117* | 0.483 ± 0.085*† |
| Tb.Sp (mm) | 0.406 ± 0.033 | 1.126 ± 0.156* | 0.219 ± 0.017* | 0.248 ± 0.023* | 0.252 ± 0.026* |
| Ct.Th (mm) | 0.366 ± 0.011 | 0.217 ± 0.013* | 0.219 ± 0.017* | 0.248 ± 0.023* | 0.252 ± 0.026* |

n = 5 per group. Values are mean ± SD.

CT, computed tomography; CON, control; IBN, ibandronate; ELD, eldecalcitol; BV/TV, bone volume/total volume; Conn.D, connectivity density; SMI, structure model index; Tb.N, trabecular number; Tb.Th, trabecular thickness; Tb.Sp, trabecular separation; Ct.Th, cortical thickness. *P < 0.05 vs. CON by Scheffe's method. †P < 0.05 vs. Vehicle by Scheffe's method.

6 months significantly improved bone strength of the proximal femur in postmenopausal women with osteoporosis (38). Bisphosphonates augment BMD through the inhibition of osteoclast-mediated bone resorption (17). Third-generation, nitrogen-containing bisphosphonates, such as IBN, act by inhibiting farnesyl diphosphate synthase in the mevalonate pathway in osteoclasts and induce apoptosis of osteoclasts, thereby inhibiting osteoclastic activity (6). On the other hand, it was reported that ELD increased BMD by suppressing the expression of receptor activator of nuclear factor kappa-B ligand in trabecular bone (12). This difference in mechanism of action on osteoclasts might be the reason for the additive effect of IBN + ELD on the increase in BMD.

A suppressive effect of IBN and/or ELD therapy on inflammation and joint destruction was not observed in the present study. Many studies have demonstrated that bisphosphonates, such as zoledronate, incadronate, and minodronate, inhibit bone and joint destruction in rat arthritis models (1, 34, 43). Several mechanisms of anti-inflammatory effects of bisphosphonates have been reported. IBN increased the number of extravasated leukocytes and had pro-inflammatory effects on the synovial microcirculation as well as induced apoptosis in synovial macrophages in mice (5, 46). However, the exact identification of target cells and interference mechanisms of bisphosphonates with the inflammatory responses are still unclear.

On the other hand, in vitro studies have shown anti-inflammatory effects of the active form of vitamin D (45), and epidemiologic analyses of patients with RA have demonstrated that vitamin D deficiency is related to inflammation, leading to increased expression of interleukin-17 and interleukin-23 (10, 15, 20). Ishikawa et al. reported that vitamin D reduced cytokine production by spleen cells in AIA rats (18). In contrast, Oelzner et al. reported that vitamin D did not affect inflammation and joint destruction in AIA rats (31).

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CONFLICTS OF INTEREST

No potential conflict of interest relevant to this arti-
cle was reported.

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