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ORIGINAL ARTICLE

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Celecoxib prevents juxta-articular osteopenia and growth plate destruction adjacent to inflamed joints in rats with collagen-induced arthritis

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Abstract The effect of celecoxib, a selective cyclooxygenase-2 inhibitor, on juxta-articular osteopenia and growth plate destruction adjacent to inflamed joints was investigated in rats with collagen-induced arthritis. Forty rats were assigned to the following six groups: (1) an untreated arthritis group; (2–5) arthritis rats receiving indomethacin (3 mg/kg per day), dexamethasone (0.2 mg/kg per day), or celecoxib (5 or 50 mg/kg per day), and (6) normal control rats. Drugs were administered for 2 weeks from the onset of arthritis. Then the hind paws were measured. Juxta-articular osteopenia and growth plate destruction adjacent to inflamed joints were also assessed using plain radiography, bone mineral density measurement, histology, and histomorphometry. Each treatment reduced inflammation, but only dexamethasone and high-dose celecoxib prevented bone loss adjacent to inflamed joints and significantly decreased bone resorption. In contrast, no treatment affected bone formation parameters. Growth plate destruction adjacent to inflamed joints was prevented by indomethacin, dexamethasone, and high-dose celecoxib. Although dexamethasone abolished inflammation, growth plate destruction was still observed. In conclusion, among the various drugs tested, only celecoxib had a preventive effect on both growth plate destruction and bone loss adjacent to inflamed joints in this arthritis model.

Key words Celecoxib · Growth plate destruction · Juvenile idiopathic arthritis · Juxta-articular osteopenia · Rheumatoid arthritis

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Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease that is characterized by synovitis associated with the progressive destruction of cartilage and bone,[1,2] including juxta-articular osteopenia adjacent to inflamed joints and focal erosion of the subchondral bone and joint margins.[3] Recent studies have suggested that both proliferating synovial cells and bone-resorbing osteoclasts play an important role in the bone resorption that occurs at these sites. Juvenile idiopathic arthritis (JIA) is another chronic inflammatory disease that is characterized by arthritis associated with progressive destruction of cartilage and bone that leads to abnormal growth of juxta-articular epiphyses, resulting in joint malalignment and destruction, extremities of different lengths, and short stature.[4]

Nonsteroidal anti-inflammatory drugs (NSAIDs), including indomethacin, are effective anti-inflammatory and analgesic agents that are commonly used to treat RA and JIA, and these drugs decrease the production of prostaglandins (PGs) by direct inhibition of the activity of cyclooxygenase (COX).[5,6] Prostaglandins (PGs) are important inflammatory mediators that are produced at sites of inflammation, including the joints of patients with RA and JIA. Prostaglandins enhance or prolong the effects of various proinflammatory agents and thus aggravate inflammation. Prostaglandins are produced from arachidonic acid by COX,[7,8] and two isoforms of COX are known to exist, which are COX-1 that is constitutively expressed by various cells and tissues[9] and COX-2 that is expressed by inflammatory cells in response to various stimuli.[10]

Previous studies on inflammatory bone conditions have showed that osteoblasts or stromal cells overexpress COX-2, which produces PGs that promote bone resorption. It was also reported that genetically COX-2-deficient mice show impaired bone resorption in response to parathyroid hormone or 1,25-hydroxyvitamin D_3. These findings suggest that COX-2 may have an important role in bone resorption as well as in inflammation, which could be quite distinct from that of COX-1.[11–13]

Selective COX-2 inhibitors are becoming more widely used to treat RA and JIA.[14] These drugs are known to reduce PG production. In addition, celecoxib (a selective COX-2 inhibitor) has been reported to have several effects on bone. Kawaguchi et al.[15] found that COX-2 inhibitors suppress osteoclastogenesis, while Igarashi et al.[16] showed that celecoxib suppresses both bone resorption and osteoclastogenesis in vitro. In addition, Katagiri et al.[17] recently reported that selective COX-2 inhibitors can reduce pannus expansion and joint erosion in a rat model of arthritis. Moreover, Mastbergen et al.[18] reported that celecoxib prevented cartilage damage induced by proinflammatory cytokines in an organ culture system.

These reports have raised the possibility that selective COX-2 inhibitors could prevent juxta-articular osteopenia and growth plate destruction in RA or JIA. Therefore, we investigated the effect of the selective COX-2 inhibitor celecoxib on juxta-articular osteopenia and growth plate destruction in rats with collagen-induced arthritis (CIA), which are commonly used as a model of inflammatory arthritis like RA and develop juxta-articular osteopenia[19] as well as early closure of the juxta-articular growth plates.[20]

Materials and methods

Collagen-induced arthritis model

Collagen-induced arthritis was induced in 6-week-old female Lewis rats (Clea Japan, Tokyo, Japan) using a modification of the method described previously.[21] Rats were anesthetized and immunized intradermally with 0.5 mg of bovine type II collagen (Cosmo Bio, Tokyo, Japan) that had been emulsified in 0.5 ml of Freund's incomplete adjuvant (Difco, Detroit, MI, USA) at 4°C. On day 7, the rats received an intradermal booster injection, which was half the volume of the first dose. The onset of arthritis in the ankle joints could usually be recognized between days 14 and 16, and animals without obvious arthritis by day 16 were excluded from this study. The incidence of arthritis was 70.0% (30 of 43 rats). On day 17, the hind paw volume of each rat was measured with a TK-101 CMP Plethysmometer (Muromachi Machine, Tokyo, Japan), and animals with a hind paw volume greater than 1.65 ml were randomized in equal numbers ($n = 5$ each) to the following five groups: (1) an untreated CIA control group, (2) a group treated with indomethacin at 3 mg/kg per day, (3) a group treated with dexamethasone at 0.2 mg/kg per day, (4) a group treated with celecoxib at 5 mg/kg per day (cele.5), and (5) a group treated with celecoxib at 50 mg/kg per day (cele.50). Five nonimmunized normal control rats were also studied according to the same experimental protocol. All present animal experiments were approved by the institutional review board of Osaka University Graduate School of Medicine.

Drug treatment

Celecoxib was a kind gift from Pharmacia (Skokie, IL, USA), while dexamethasone and indomethacin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Drugs were prepared as suspensions in 0.5% methylcellulose (Wako, Tokyo, Japan). Rats were treated orally once a day for 2 weeks at the above-mentioned doses with a dosing volume of 0.5 ml/day. Administration was begun on day 17 of the study and continued until the final assessment on day 30.

Radiographic evaluation

On day 30, all rats underwent radiography. After being killed with an overdose of ketamine intramuscularly, the lower extremities were resected and the bones were cleaned of adherent tissue. Then the limbs were positioned over a cassette containing X-ray film (Eastman-Kodak, Seattle, WA, USA) and radiographs were obtained with a conventional microradiography unit (M-60, Softex, Tokyo, Japan) at 30 kV and 3 mA for 75 s.

Measurement of bone mineral density (BMD)

The BMD of the proximal one-third of the tibia was measured by a bone densitometer (Lunar PIXImus; Lunar, Madison, WI, USA) using software provided with the instrument. The region-of-interest (ROI) tool was employed to identify the proximal tibia. To eliminate the fibula from the scans, the oval exclusion ROI was positioned over this bone (Fig. 3Aa).[22] All BMD analyses were done by the same investigator (H.T.).

Bone histomorphometry

All rats underwent double fluorescent labeling before being euthanized. On days 23 and 27, tetracycline hydrochloride (20 mg/kg; Sigma) were injected intraperitoneally. Bone specimens were fixed in 70% ethanol, prestained in Villanueva bone stained for 72 h, dehydrated in alcohol and acetone, and embedded in methylmethacrylate. Then the proximal tibia was cut into 5-μm thick frontal sections for histomorphometry of cancellous bone.[23] Measurements were performed at a magnification of ×320 in the secondary spongiosa at 1 mm from the growth plate using Bone Histomorphometric System software (System Supply, Nagano, Japan). The histomorphometric parameters employed in this study were derived from Parfitt et al., and have been approved by an American Society for Bone and Mineral Research (ASBMR) committee.[24] As static parameters, the trabecular bone volume (BV/TV) and trabecular thickness (Tb.Th) were measured. To measure bone formation, the osteoid surface relative to bone surface and the osteoblast surface relative to bone surface were calculated (OS/BS and Ob.S/BS, respectively). To assess bone resorption, the eroded surface and osteoclast surface were quantified

relative to the bone surface (ES/BS and Oc.S/BS, respectively). To determine the effect of each drug on growth plate destruction, the width of the growth plate and the number of osteoclasts relative to the bone surface (N.Oc/BS) were measured.

Statistical analysis

Results are presented as the mean ± SD. Differences were analyzed by using analysis of variance, and $P < 0.05$ was considered to indicate statistical significance.

Results

Hind paw volume

The hind paw volume of the rats was measured with a TK-101 CMP Plethysmometer at weekly intervals. On days 24 and 30, the hind paw volume of drug-treated animals was significantly smaller than that of the untreated CIA control group (Fig. 1).

Juxta-articular osteopenia

Radiography of the knee joint showed that juxta-articular osteopenia was very mild in the cele.50 or dexamethasone groups, while severe juxta-articular osteopenia was seen in the untreated CIA, indomethacin, and cele.5 groups (Fig. 2).

Quantitative evaluation of juxta-articular osteopenia by measuring the BMD of the proximal one-third of the tibia (Fig. 3Aa) showed that BMD was significantly higher in the cele.50 group (mean ± SD: 0.183 ± 0.02 g/cm^2) and the dexamethasone group (0.247 ± 0.09 g/cm^2) than in the untreated CIA rats (0.155 ± 0.05 g/cm^2). However, there was no significant difference of BMD between the indomethacin group (0.155 ± 0.01 g/cm^2) or the cele.5 group (0.166 ± 0.02 g/cm^2) and the untreated CIA rats (0.155 ± 0.05 g/cm^2) (Fig. 3Ab). Histomorphometric analysis gave results consistent with the BMD data because decrease of BV/TV and Tb.Th were significantly suppressed in the cele.50 group (13.6% ± 3.15% and 48.1 ± 5.43 μm, respectively) and the dexamethasone group (22.8% ± 6.43% and 56.9 ± 4.56 μm) than in the untreated CIA group (5.32% ± 1.53% and 34.0 ± 5.14 μm). This protective effect of high-dose celecoxib and dexamethasone against bone loss in CIA rats was accompanied by a significant decrease of ES/BS (cele.50 and dexamethasone: 20.6% ± 6.37% and 11.6% ± 3.33%, respectively) and Oc.S/BS (6.92% ± 3.02% and 3.43% ± 1.39%), which are bone resorption parameters, compared with the untreated CIA group (ES/BS 39.4% ± 4.94% and Oc.S/BS 13.5% ± 5.40%). However, there were no significant changes of bone formation parameters (OS/BS and Ob.S/BS were respectively 28.3% ± 17.2% and 19.7% ± 15.5% for cele.50, 30.4% ± 5.93% and 16.4% ± 3.36% for dexamethasone, and 27.2% ± 12.3% and 17.9% ± 10.8% for untreated CIA). On the other hand, there were no significant differences of any parameters between the indomethacin group (BV/TV, Tb.Th, ES/BS, Oc.S/BS, OS/BS, and Ob.S/BS were 6.64% ± 0.636%, 33.1% ± 1.38 μm, 44.4% ± 0.226%, 18.2% ± 0.58%, 13.4%

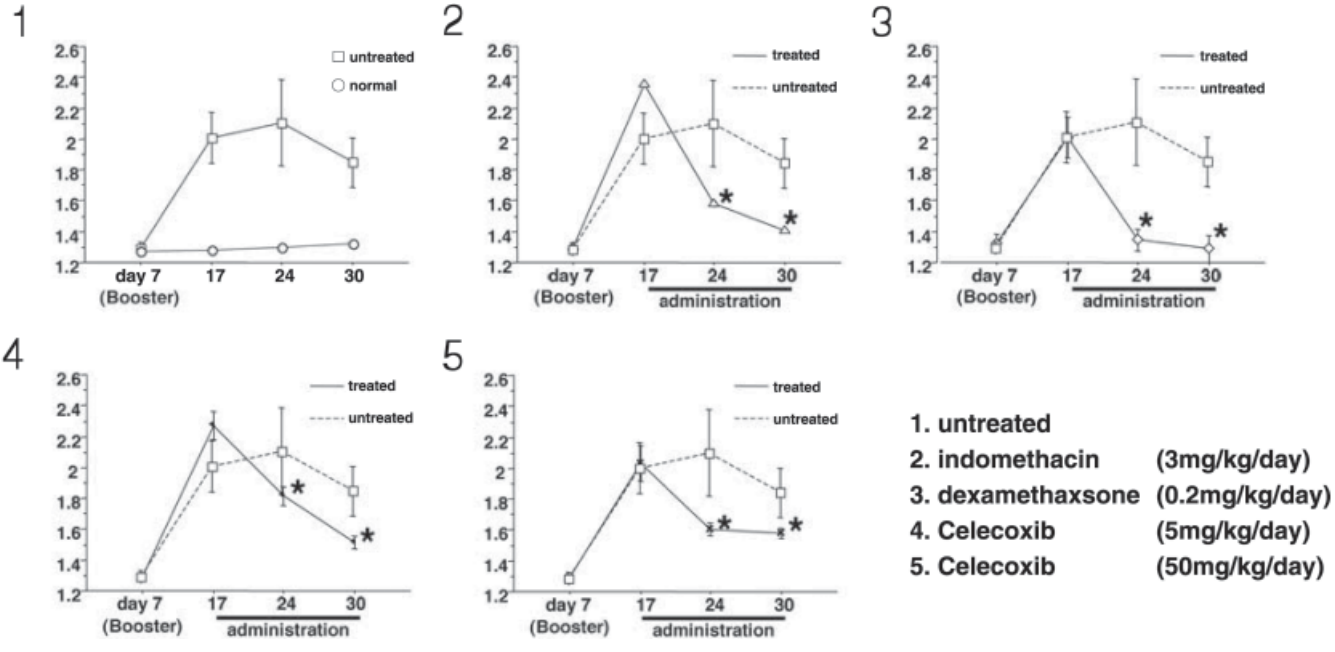

Fig. 1. Effect on the hind paw volume. Each drug significantly improved collagen-induced arthritis (CIA), as evaluated by hind paw volume. Data from five rats in each group were presented as the mean ± SD. *1*, untreated CIA group; *2*, indomethacin group; *3*, dexamethasone group; *4*, cele.5 group; *5*, cele.50 group; *6*, normal control group. On days 24 and 30, the footpad volume in groups 2, 3, 4 and 5 was significantly smaller than in the untreated CIA control group (1). *$P < 0.01$ for the untreated CIA control group vs. each treated group (analysis of variance; ANOVA)

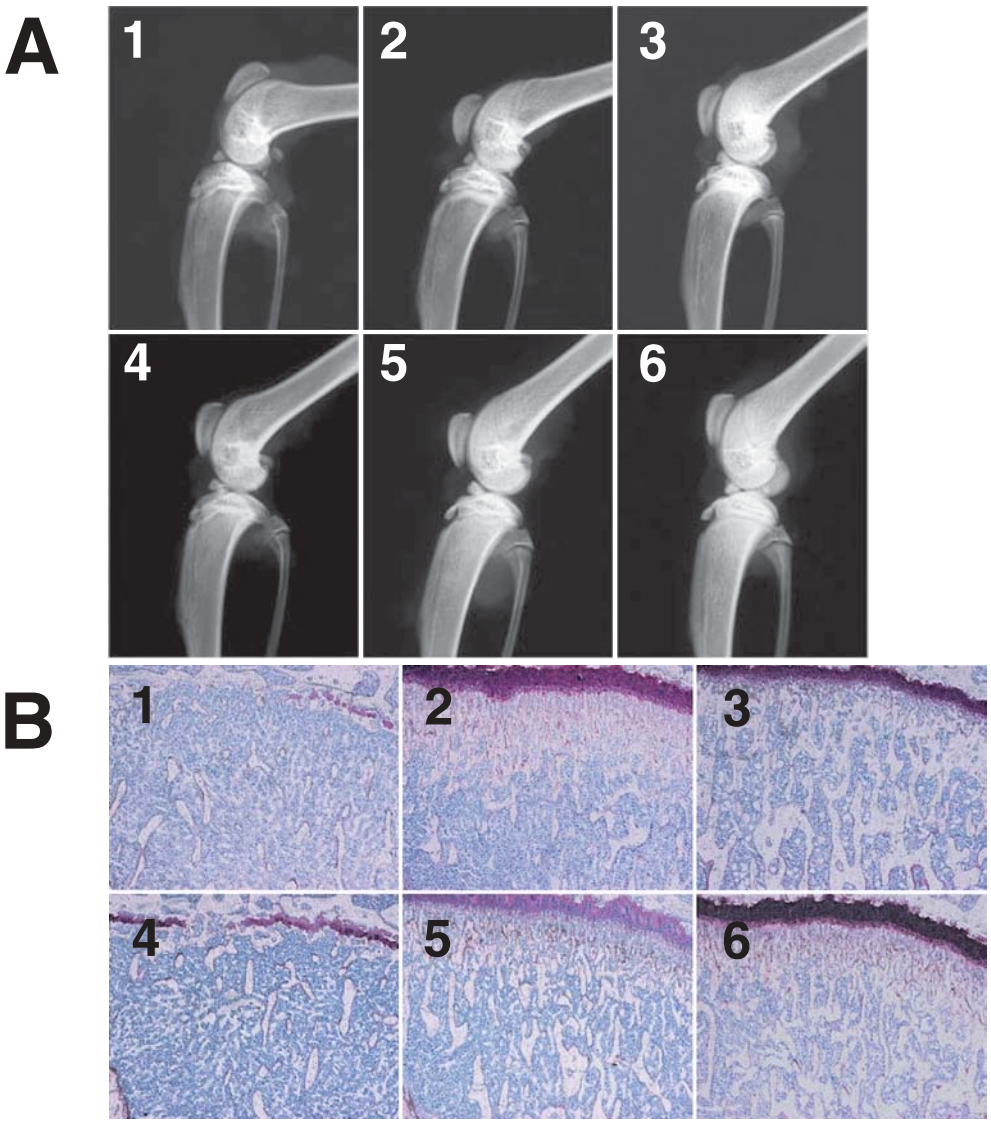

Fig. 2A,B. Effect on juxta-articular osteopenia. **A** Radiographic findings. Representative images selected from the five rats (ten limbs) in each group are presented. *1*, untreated CIA group; *2*, indomethacin group; *3*, dexamethasone group; *4*, cele.5 group; *5*, cele.50 group; *6*, normal control group. Rats from the cele.50 group (5) and the dexamethasone group (3) showed denser juxta-articular bone than untreated CIA rats (1) or rats from the indomethacin group (2) or the cele.5 group (4). An X-ray image of a nonimmunized normal control rat is also shown (6). **B** Histological villanueva bone stained findings. Representative images from the five rats (ten specimens) in each experimental group are presented. Specimens from the untreated CIA group (*1*), the indomethacin group (*2*), and the cele.5 group (*4*) reveal marked loss of bone trabeculae at the proximal tibial epiphysis, as well as the metaphysic, when compared with the nonimmunized normal control rats (*6*). In the cele.50 group (*5*) and the dexamethasone group (*3*), trabeculae are also decreased compared with the non-immunized normal control group (*6*), but are preserved to a certain extent. Original magnification ×40

± 5.43%, and 8.61% ± 5.16%, respectively) or the cele.5 group (6.48% ± 0.757%, 37.0 ± 1.68 µm, 40.3% ± 6.24%, 19.1% ± 6.27%, 24.7% ± 9.21%, and 14.4% ± 9.69%) and the untreated CIA rats. Bone histomorphometry revealed the following values in normal control rats. BV/TV, Tb.Th, ES/BS, Oc.S/BS, OS/BS, and Ob.S/BS were 28.3% ± 3.46%, 55.9 ± 4.65 µm, 29.7% ± 3.00%, 12.5% ± 1.01%, 31.1% ± 5.9%, and 22.2% ± 5.21%, respectively.

Growth plate thickness

Histological examination showed that the growth plate was almost completely preserved in the indomethacin and cele.50 groups, and was partially preserved in the dexamethasone group. In contrast, the growth plate had disappeared in the untreated CIA rats (Fig. 4A, B). Quantitative evaluation of the width of the growth plate showed no significant difference between normal control rats (129 ± 10.7 mm) and the indomethacin group (126 ± 14.1 mm) or the cele.50 group (136 ± 47.7 mm). However, a significant difference was noted between normal rats and the dexamethasone group (61.9 ± 10.0 mm), the cele.5 group (15.2 ± 4.64 mm), and the untreated CIA group (16.1 ± 15.2 mm). The value of N.Oc/BS before administration (on day 17) and in the untreated, indomethacin, dexamethasone, cele.5, and cele.50 groups was 3.21 ± 0.636 no./mm, 2.79 ± 0.636 no./mm, 3.74 ± 0.87 no./mm, 0.640 ± 0.256 no./mm, 4.96 ± 1.92 no./mm, and 1.02 ± 0.694 no./mm, respectively. N.Oc/BS showed a significant difference between before administration (day 17) and the values obtained in the dexamethasone group and the cele.50 group.

Discussion

In this study, we demonstrated an anti-inflammatory effect of high-dose celecoxib (50 mg/kg per day), as well as an inhibitory effect on juxta-articular osteopenia, which was mainly due to decreased bone resorption according to the histomorphometric findings. High-dose celecoxib also pre-

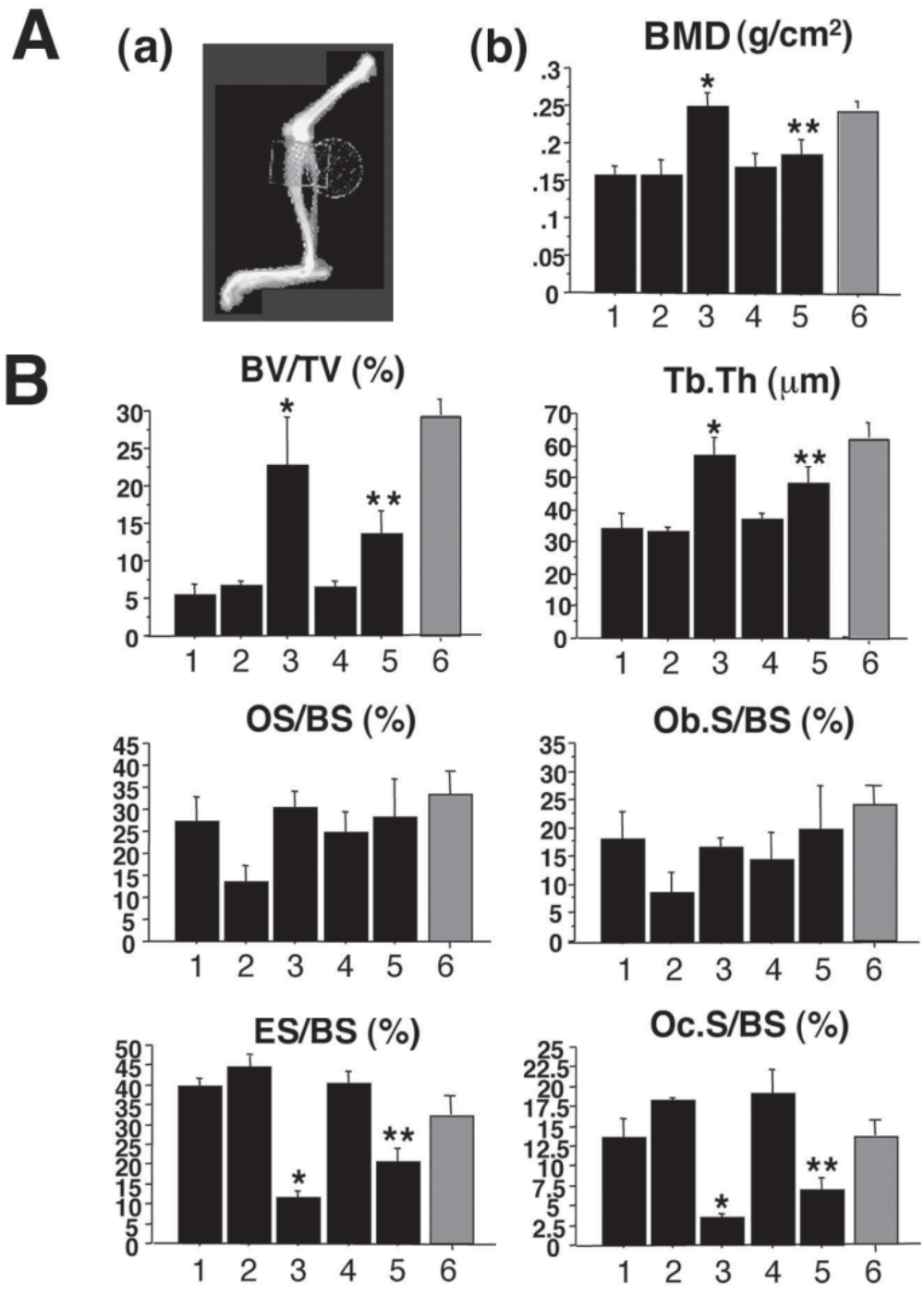

Fig. 3A,B. Effect on the bone mineral density (*BMD*) and histomorphometric parameters. **A** BMD analysis. (**a**) The BMD of the proximal one-third of tibia was measured by a bone densitometer using special software provided by the manufacturer. The region-of-interest tool was employed to define the proximal tibia and to eliminate the fibula from the scans. (**b**) Data are shown as the mean ± SD for five rats in each experimental group: *1*, untreated CIA group; *2*, indomethacin group; *3*, dexamethasone group; *4*, cele.5 group; *5*, cele.50 group; *6*, normal control group. $*P < 0.01$, $**P < 0.05$ for the untreated CIA group vs the dexamethasone and cele.50 groups, respectively (ANOVA). **B** Histomorphometric analysis. *1*, untreated CIA group; *2*, indomethacin group; *3*, dexamethasone group; *4*, cele.5 group; *5*, cele.50 group; *6*, normal control group. Both BV/TV and Tb.Th were significantly preserved in the cele.50 group (*5*) and the dexamethasone group (*3*). $*P < 0.01$, $**P < 0.05$ for the untreated CIA group vs the dexamethasone group and cele.50 group, respectively (ANOVA). *BV/TV*, trabecular bone volume; *Tb.Th*, trabecular thickness; *OS/BS*, osteoid surface relative to bone surface; *Ob.S/BS*, osteoblast surface relative to bone surface; *ES/BS*, eroded surface quantified relative to the bone surface; *Oc.S/BS*, osteoclast surface quantified relative to the bone surface

vented the growth plate destruction that usually occurs in CIA. In contrast, low-dose celecoxib (5 mg/kg per day) did not demonstrate a preventive effect on juxta-articular osteopenia or growth plate destruction, although it still reduced inflammation. These findings suggest that celecoxib may have different actions at high doses from those seen at low doses. The low dose of celecoxib used in this study is similar to the clinical dosage for RA patients and there have been no previous reports about the improvement of juxta-articular osteopenia and growth plate destruction by celecoxib in either the experimental or clinical setting. Therefore, further investigations will be necessary to better define this anti-osteoporotic effect of celecoxib, including studies on juxta-articular osteopenia and growth plate destruction in patients with RA and JIA.

Animals with CIA, including rats and mice, are commonly used to investigate the pathology of inflammatory arthritides such as RA or to confirm the effects of anti-inflammatory drugs. Previously, Bogoch et al.[19] reported the occurrence of juxta-articular bone loss in rabbits with experimental inflammatory arthritis. Hanyu et al.[25] concluded that the cause of juxta-articular osteopenia in CIA rats was a significant increase of osteoclasts along with a decrease in the rate of bone formation based on their histomorphomet-

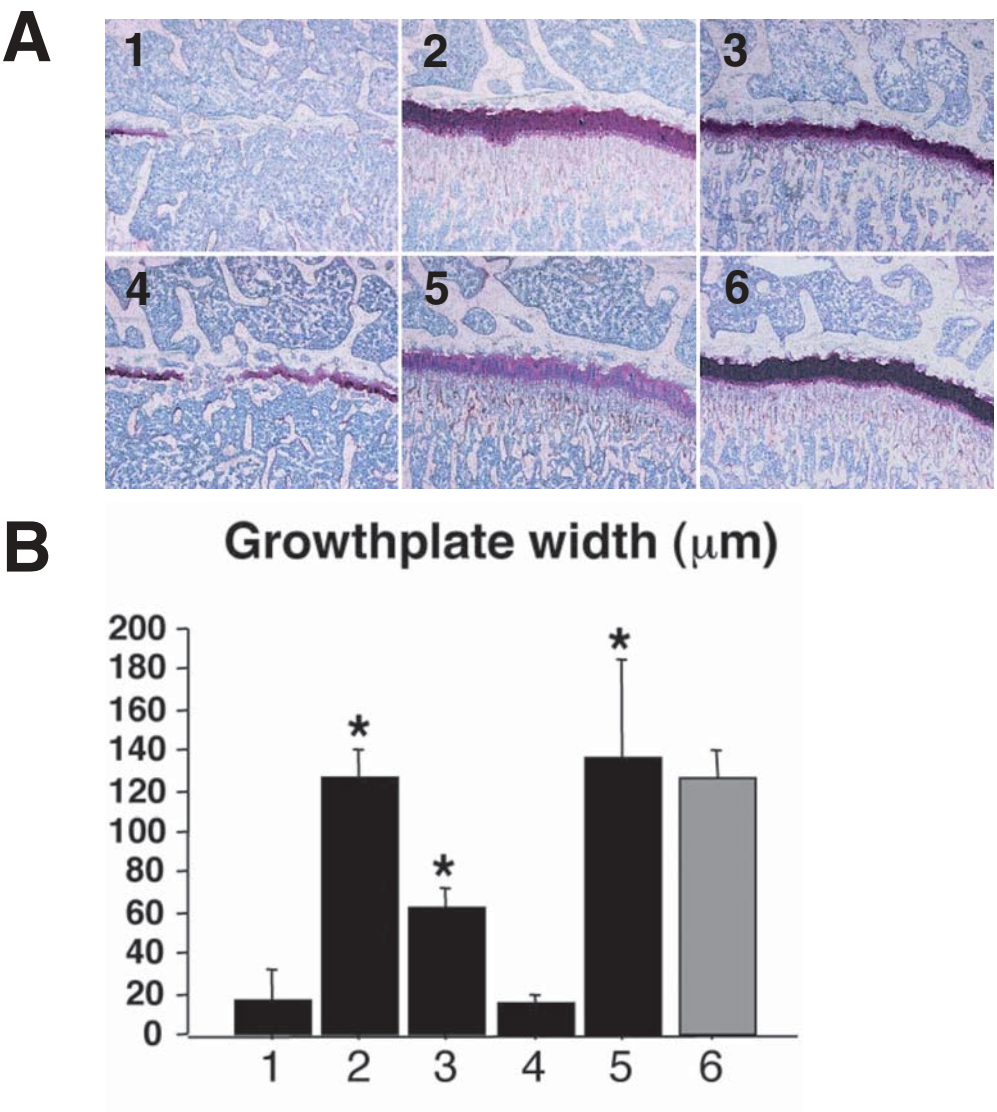

Fig. 4A,B. Effect on growth plate destruction. **A** Histological findings in the *1*, untreated CIA group; *2*, indomethacin group; *3*, dexamethasone group; *4*, cele.5 group; *5*, cele.50 group; *6*, normal control group. **B** Growth plate width. Measurement of the proximal tibial growth plate showed that it was significantly wider in the indomethacin, dexamethasone, and cele.50 groups than in the untreated CIA group or cele.5 group. Both indomethacin and high-dose celecoxib were more effective than dexamethasone. *$P < 0.01$ for untreated CIA group vs the indomethacin, dexamethasone, and cele.50 groups (ANOVA)

ric analysis. Juxta-articular osteopenia is a typical finding in RA patients, and osteoclasts are often observed in the juxta-articular region on histological examination.[1,26–30] Accordingly, osteoclasts are thought to play an important role in the occurrence of juxta-articular osteopenia in patients with RA, especially when there is rapid bone erosion. Hanyu et al.[25] also reported a decrease in the rate of bone formation in CIA rats. Our histomorphometric analysis demonstrated that the protective effect of high-dose celecoxib against bone loss in CIA rats was associated with a significant decrease of bone resorption by osteoclasts. However, high-dose celecoxib could not ameliorate the decrease of bone formation parameters in CIA, indicating that decreased bone resorption by osteoclasts may be the major action by which high-dose celecoxib prevents bone loss.

Recently, high-dose celecoxib was reported to induce apoptosis of cancer cells, stromal cells, and rheumatoid synovial fibroblasts, although low doses do not have an apoptogenic effect.[31–35] This was an unexpected finding for a COX-2 inhibitor, and it seems to be a specific action of celecoxib alone.[31–35] Accordingly, osteoclastogenesis in the juxta-articular region might have been suppressed by decreased RANKL expression on fibroblasts or stromal cells, which is essential for osteoclastogenesis to occur,[26–30] through both the inhibition of PG production and an apoptotic effect on these cells. Therefore, juxta-articular osteopenia may have been improved by a decrease of osteoclasts as our bone histomorphometric analysis showed. Furthermore, our data suggest that high-dose celecoxib may not only prevent inflammation but also juxta-articular osteopenia in patients with RA.

Although low-dose celecoxib and indomethacin had an excellent anti-inflammatory effect, neither agent improved juxta-articular osteopenia in CIA rats. Thus, the low dose of celecoxib and the dose of indomethacin that we tested (a common clinical dose) may have been insufficient to inhibit osteoclastogenesis in CIA rats. It has already been reported that methotrexate and dexamethasone can inhibit joint destruction in rats with arthritis,[36–38] but there have been no studies showing that NSAIDs including COX-2 inhibitors could prevent juxta-articular osteopenia. It may be necessary to suppress disease activity more strongly to prevent

juxta-articular osteopenia in RA patients. The present results also imply that the usual dose of celecoxib may be too low to prevent juxta-articular osteopenia in RA patients, although it is effective against inflammation. Because high-dose celecoxib could reduce juxta-articular osteopenia in CIA rats, high-dose therapy may be a possible new modality to prevent juxta-articular osteopenia in patients with RA.

In the present study, we demonstrated that indomethacin and high-dose celecoxib almost completely prevented early growth plate closure in CIA rats, while dexamethasone partially prevented growth plate destruction. We previously reported[20] that early closure of epiphyseal growth plates led to poor development of the long bones in CIA rats and showed that overexpression of matrix metalloproteinase-3 (MMP-3), which may be involved in proteoglycan degradation, and vascular endothelial growth factor (VEGF), which is associated with cartilage ossification and angiogenesis, might play a role. Therefore, VEGF may be involved in causing an increase of osteoclasts/chondroclasts, which results in destruction of the growth plate. Our histomorphometric analysis demonstrated a decrease of osteoclasts in the dexamethasone group and the cele.50 group, findings that may explain one of the mechanisms preventing growth plate destruction. Abdelrahim and Safe[39] reported that COX-2 inhibitors decrease VEGF expression by colon cancer cells, while Sanchez et al.[40] reported that NSAIDs (including COX-2 inhibitors) did not alter MMP-3 production by cultured human chondrocytes. These finding may also help to explain the mechanism by which celecoxib prevented growth plate destruction in CIA rats. On the other hand, although dexamethasone completely abolished paw swelling and juxta-articular osteopenia, it only had a limited preventive effect on growth plate destruction. It was recently reported that dexamethasone can damage the growth plate in rats by causing apoptosis of growth plate chondrocytes.[41] Therefore, the dose of dexamethasone that suppresses arthritis may concurrently have an adverse influence on the growth plate in rats. A decrease of BMD is common in children and adolescents with JIA, resulting in reduced bone mass and a higher risk of osteoporosis.[42] Taking this point into consideration, it is not only important to reduce inflammation but also bone loss in patients with JIA. High-dose celecoxib may be a new candidate to prevent juxta-articular osteopenia in patients with JIA as well as RA, while also maintaining growth plate integrity in JIA patients whose growth plates are still open.

In conclusion, our findings suggested that a selective COX-2 inhibitor, celecoxib, is not only effective against inflammation, but also prevents juxta-articular osteopenia adjacent to inflamed joints in rats with CIA. Moreover, this drug prevents destruction of the growth plates adjacent to inflamed joints, which occurs in JIA and leads to premature growth plate closure with resultant epiphyseal deformity.

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