Effects of the selective EP$_4$ antagonist, CJ-023,423 on chronic inflammation and bone destruction in rat adjuvant-induced arthritis

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Abstract

Prostaglandin E$_2$ (PGE$_2$) produced by cyclooxygenase (COX) is a potent pro-inflammatory mediator. We have recently discovered CJ-023,423, a highly selective antagonist of EP$_4$ receptors, one of the PGE$_2$ receptors. This agent is suitable for exploring the effects of blocking EP$_4$ receptors following oral administration in rats. In this study, CJ-023,423 was used in rats with adjuvant-induced arthritis (AIA) to investigate the role of the EP$_4$ receptor in chronic inflammation and bone destruction. These effects were compared with those of rofecoxib, a selective COX-2 inhibitor. CJ-023,423 had significant inhibitory effects on paw swelling, inflammatory biomarkers, synovial inflammation and bone destruction in AIA rats. In particular, the inhibitory effect on paw swelling in AIA rats was comparable to that of rofecoxib. These results suggest that PGE$_2$ acting via the EP$_4$ receptor is involved in the development of chronic inflammation and bone destruction, particularly with respect to oedema in AIA rats. This is the first study to confirm the in-vivo effects of EP$_4$ receptor blockade on inflammation and bone destruction in AIA rats with a small-molecule compound.

Introduction

Prostaglandin E$_2$ (PGE$_2$) is well known as a pro-inflammatory lipid mediator produced by cyclooxygenase (COX), and the pathways following PGE$_2$ stimulation have been clarified. PGE$_2$ receptors are divided into four subtypes: EP$_1$, EP$_2$, EP$_3$ and EP$_4$; activation of these receptors by PGE$_2$ is transduced by the activation of G-proteins. Each receptor subtype has a distinct signal pathway and physiological function evoked by PGE$_2$ (Coleman et al 1994; Narumiya et al 1999; Breyer et al 2001). EP$_1$ receptors are coupled to an increase in intracellular calcium ion concentration. The EP$_2$ and EP$_4$ receptors are coupled to stimulation of adenylate cyclase via the Gs protein and an increase in intracellular cyclic AMP (cAMP) (Campagnuolo et al 2002). The EP$_3$ receptor inhibits adenylate cyclase via the Gi protein, leading to a decrease in the intracellular cAMP. Regarding the role of the EP$_4$ receptor in PGE$_2$-mediated inflammation, it was reported that EP$_4$-receptor-deficient mice showed decreases in both the incidence and severity of collagen-induced arthritis compared with wild-type mice and EP$_1$-, EP$_2$- and EP$_3$-deficient mice (McCoy et al 2002). On the other hand, intra-articular injection of the EP$_4$ agonist ONO-AE1-329 suppressed hind-paw swelling in rats injected with complete Freund's adjuvant (CFA) (Omote et al 2002). Thus, sharply contrasting results have been reported in terms of whether antagonism or stimulation of EP$_4$ receptors exhibits anti-inflammatory effects.

Adjuvant-induced arthritis (AIA) is an experimental rat polyarthritis model, which has several features that resemble rheumatoid arthritis in patients, including joint swelling, pannus formation, joint destruction and bone erosion. In order to assess the anti-inflammatory effect in this model, hind-paw swelling and levels of serum biomarkers such as sialic acid and the albumin/globulin (A/G) ratio have been generally used (Kourounakis et al 1991; Tanahashi et al 1998). Continuous administration of non-steroidal anti-inflammatory agents, including cyclo-oxygenase (COX)-2 inhibitors, has been shown to affect paw swelling, inflammatory cell infiltration, pannus formation and bone destruction (Taurog et al 1988; Anderson et al 1996; Van Eden & Waksman

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2003). Among mediators produced by COXs, PGE_2 is known to be a principal pro-inflammatory mediator in AIA rats, because neutralizing anti-PGE_2 antibody suppressed paw swelling to the same extent as indometacin (Portanova et al 1996). Recently, the mechanism of bone destruction by osteoclast activation was clarified in this model (Schett et al 2003). In addition, a few reports indicated that EP_4 antagonists suppress osteoclast formation in in-vitro experiments using mice bone-marrow cells (Ono et al 1998; Tomita et al 2002; Takita et al 2007). It would be interesting to know the contribution of EP_4 to synovial inflammation and bone destruction in AIA rats.

We have recently discovered CJ-023,423 (*N*-[({2-[4-(2-ethyl-4,6-dimethyl-1*H*-imidazo [4, 5-*c*] pyridin-1-yl) phenyl] ethyl} amino) carbonyl]-4-methylbenzenesulfonamide), a novel, potent and selective EP_4 antagonist (Nakao et al 2007). CJ-023,423 is highly selective for the human EP_4 receptor compared with other human prostanoid receptors (i.e. EP_1, EP_2, EP_3, DP, FP, IP and TP). Orally administrated CJ-023,423 reduced inflammatory pain such as carrageenan-induced mechanical hyperalgesia and CFA-induced weight-bearing deficit in rats. Therefore, CJ-023,423 is a useful tool to investigate the contribution of the EP_4 receptor in in-vivo disease aetiology.

In this study, we evaluated the effects of CJ-023,423 on paw swelling, inflammatory biomarkers, synovial inflammation and bone erosion in AIA rats, in order to clarify the involvement of the EP_4 receptor in the pathogenesis of chronic inflammation and bone destruction. These effects were compared with those of rofecoxib, a COX-2 selective inhibitor.

Materials and Methods

Materials

The sodium salt of CJ-023,423 and rofecoxib were synthesized by Pfizer Global Research and Development (PGRD; Aichi, Japan). *Mycobacterium tuberculosis* H37 RA was purchased from Difco Laboratories (Detroit, MI, USA). Liquid paraffin and methylcellulose (MC) were purchased from Wako Pure Chemical Industries (Osaka, Japan). The sialic acid assay kit was purchased from Kyokuto Pharmaceutical Industrial Co. (Tokyo, Japan).

Animals

All procedures used in the in-vivo assays were approved by the Animal Ethics Committee at the PGRD Nagoya Laboratories (Japan) and were performed according to the International Laboratory Animal Welfare Guidelines.

Male Lewis rats (170–190 g) were purchased from Charles River (Hino, Japan). Animals were housed in pairs in steel-wire cages, with free access to food and water. The animals were kept under conditions of constant temperature ($23 \pm 2°C$) and humidity ($55 \pm 15\%$) with a 12 h light–dark cycle (lights on 07:00). Animals were housed under these conditions for 4–5 days before the start of the experiments.

Adjuvant arthritis

Induction of AIA and drug treatment

Rats were grouped into eight groups according to body weight on the day before adjuvant injection. The groups were: the disease-control group, CJ-023,423 groups, treated with 29, 57 or 96 mg kg^{-1}, twice daily, rofecoxib groups, treated with 0.5, 1.5 or 5 mg kg^{-1} twice daily, and the normal-control group. All rats except for the normal-control group were given a subcutaneous injection of 600 μg of *M. tuberculosis* H37 RA suspended in 100 μL liquid paraffin into the right hind footpad on day 0 at 14:00. CJ-023,423 and rofecoxib were suspended in 0.1% MC (4000 cp) and administered orally at 08:30 and 18:30 from day 0 to day 21 (for 22 days) in a volume of 1 mL per 100 g body weight. Rats in the disease- and normal-control groups were given drug vehicle (0.1% MC, 1 mL per 100 g body weight, twice daily). Drug/vehicle administration was started on day 0 and continued until final assessment on day 21. Rats in the normal-control group were given vehicle only from day 0 to day 21. Body weight and hind-paw swelling were measured intermittently during this period. After the final assessment, rats were fasted overnight, and the next day were anaesthetized with pentobarbital to allow collection of blood via the descending aorta into a Separapait tube (Sekisui Chemical, Osaka, Japan), after which they were euthanized.

Assessment of arthritis

The hind paw volumes of the ipsilateral and contralateral paws were measured using a plethysmometer (Unicom, Chiba, Japan) on the days before adjuvant injection and on days 1, 4, 6, 9, 13, 16, 19 and 21.

Serum was separated from the blood collected on day 22 by centrifugation. Sialic acid and the A/G ratio were measured as inflammatory biomarkers in the serum. The amount of sialic acid in the serum was determined using a commercial kit; albumin and globulin were measured using an automatic analyser (Hitachi 7070, Tokyo, Japan).

Amputated hind paws were fixed with 4% paraformaldehyde, decalcified with 10% EDTA, embedded into paraffin, and sectioned and stained with haematoxylin and eosin (H&E) for histopathological evaluation. Two components of the arthritic process in the tarsal joint were evaluated for each paw using semiquantitative grading criteria (shown in Table 1) according to procedures described previously (Campagnuolo et al 2002). Measures of synovial inflammation, including inflammatory cell infiltration, fibrosis and abscess formation, in periarticular soft tissues, and bone destruction were acquired with a blinded analytical paradigm.

Data analysis

Data are presented as mean $\pm$ s.e.m. or median with first and third quartiles. Differences between treatment groups were tested by one-way analysis of variance with Dunnett's test, or unpaired two-tailed Student's *t*-test. Histopathological lesion scores were analysed using the Kruskal–Wallis test with Dunn's multiple comparison test or rank sum test. *P* values of less than 0.05 at a 95% confidence level were considered significant.

Table 1 Histopathology criteria for scoring lesions in rats with adjuvant-induced arthritis (from Campagnuolo et al (2002))

Score	Feature
Synovial inflammation	
0	Normal
1	Mild inflammation
2	Moderate inflammation (often, but not always, not diffuse)
3	Moderate inflammation (often, but not always, diffuse)
4	Marked inflammation (diffuse and dense, with synovial abscesses or diffuse fibrosis)
Bone destruction	
0	Normal
1	Minimal loss of cortical or trabecular bone at rare sites
2	Mild loss of cortical or trabecular bone at rare sites
3	Moderate loss of bone at many sites (usually the trabeculae of the tarsals, but sometimes the cortex of the distal tibia)
4	Marked loss of bone at many sites (usually as extensive destruction of trabeculae in the tarsals, but sometimes with partial loss of cortical bone in the distal tibia)
5	Marked loss of bone at many sites (with fragmenting of tarsal trabeculae and full thickness penetration of cortical bone in the distal tibia)

Results and Discussion

Effects of CJ-023,423 and rofecoxib on hind-paw swelling

Paw swelling in the ipsilateral paw developed immediately after adjuvant injection and increased until day 21, reaching a volume of more than 4 mL on day 21 (Figure 1A and C). On the other hand, contralateral paw swelling developed 13 days after adjuvant injection, and increased to 3 mL on day 21 (Figure 1B and D). The average volumes of both hind paws in the normal rats were almost 1.8 mL during the experimental period.

Prophylactic oral administration of either CJ-023,423 (29, 57 or 96 mg kg^{-1} twice daily) or rofecoxib (0.5, 1.5 or 5 mg kg^{-1} twice daily) was started on day 0 and continued until day 21. Both compounds significantly inhibited swelling of the ipsilateral (Figure 1A and C) and contralateral paws (Figure 1B and D) compared with the disease-control group. The inhibitory effects of CJ-023,423 and rofecoxib on contralateral paw swelling reached a plateau above 57 and 1.5 mg kg^{-1} twice daily, respectively.

Effects on inflammatory biomarkers

To investigate the systemic anti-inflammatory effects of CJ-023,423, serum sialic acid and A/G ratio were measured on day 22. As shown in Table 2, serum sialic acid in AIA rats significantly increased to 1.86 mg mL^{-1}, compared with 0.9 mg mL^{-1} in the normal-control group. CJ-023,423 significantly suppressed serum sialic acid in a dose-dependent manner, and the effect reached a plateau over 57 mg kg^{-1} twice daily. Rofecoxib also significantly suppressed serum sialic acid in a dose-dependent manner.

The A/G ratio decreased significantly in AIA rats to 2.35, compared with 3.04 in the normal-control group (Table 2). CJ-023,423 and rofecoxib significantly improved the A/G ratio compared with the disease-control group, in a dose-dependent manner.

Effects of CJ-023,423 and rofecoxib on histopathological evaluation in AIA rats

Figures 2 and 3 show photomicrographs of tarsal joint sections stained with H&E in normal-control, disease-control (AIA) and compound-treated rats. No inflammation or tissue destruction was seen in normal-control rats (Figures 2A, 3A, 3C). In contrast, the adjuvant-injected ipsilateral tarsal joints of disease-control rats showed severe joint destruction with extensive inflammation and erosion of cartilage and bone on day 22 (Figure 2B). The joint cavity was filled with infiltrated neutrophils (Figure 3B). Inflammation extended to the dermis and included severe oedema, proliferation of fibroblasts, many inflammatory cells such as neutrophils, macrophages and lymphocytes, and abscess formation. There were many newly formed immature bone trabeculae at the erosion sites of bone, with large numbers of activated osteoblasts and osteoclasts (Figure 3D) on and around them. In addition, unaffected marrow space was hypercellular (increased haematopoiesis) compared with normal rats. These signs of inflammation and bone destruction were reduced in rats treated with CJ-023,423 96 mg kg^{-1} twice daily (Figure 2C) or rofecoxib 5 mg kg^{-1} twice daily (Figure 2D), Subcutaneous oedema was reduced in the drug-treated groups in a dose-dependent manner.

To evaluate the ability of these compounds to suppress synovial inflammation in periarticular soft tissues and bone destruction, the severity of the lesions was assessed using semiquantitative grading criteria (Table 1) (Campagnuolo et al 2002). Table 3 shows the synovial inflammation score and the bone destruction score. Synovial inflammation scores of both tarsal joints in disease-control rats reached 4.0 points (normal-control rats: 0 points). CJ-023,423 significantly reduced synovial inflammation scores in both joints in a dose-dependent manner, and the effect reached a plateau above 57 mg kg^{-1} twice daily. Rofecoxib also significantly reduced the synovial inflammation score in both joints in a dose-dependent manner. The bone destruction score for both joints in AIA rats reached 5 points (normal-control rats: 0 points),
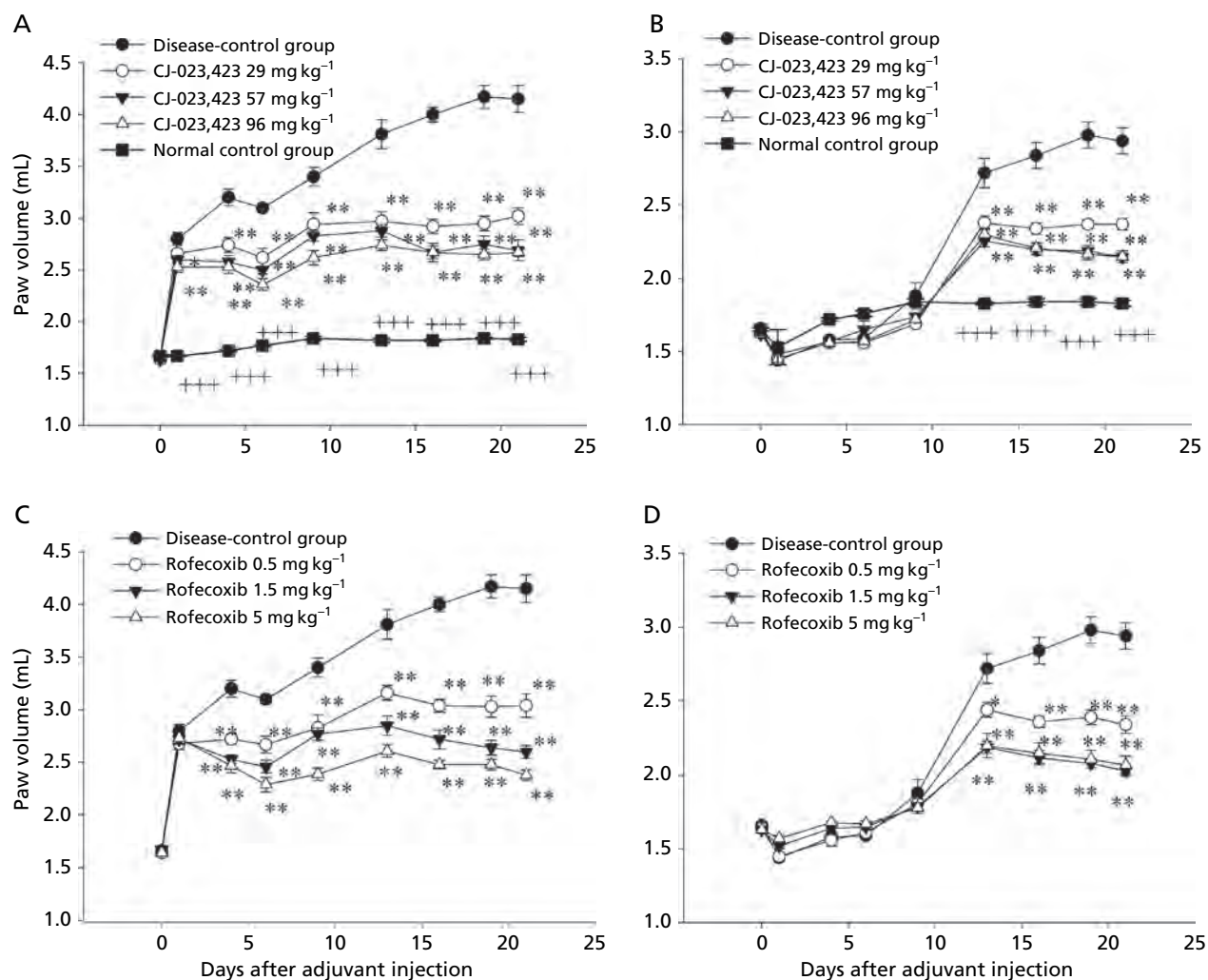
Figure 1 Inhibitory effects of CJ-023,423 and rofecoxib on paw swelling in rats with adjuvant-induced arthritis. Swelling of the ipsilateral (A, C) and contralateral hind paws (B, D) was measured with a plethysmometer on days −1, 1, 4, 6, 9, 13, 16, 19 and 21. Rats were treated orally with CJ-023,423 (29, 57 or 96 mgkg^{-1}), rofecoxib (0.5, 1.5 or 5 mgkg^{-1}) or vehicle twice daily from day 0 to day 21. Data are mean ± s.e.m for eight rats. *$P < 0.05$; **$P < 0.01$ vs disease-control group (one-way analysis of variance followed by Dunnett's test; $^{+++}P < 0.001$ vs disease-control group (Student's unpaired t-test).

as shown in Table 3. CJ-023,423 significantly reduced the bone destruction score on the ipsilateral joint, and the effect reached a plateau above 57 mgkg^{-1} twice daily. Rofecoxib also significantly suppressed bone destruction in the ipsilateral and contralateral joints in a dose-dependent manner.

The pharmacokinetics (PK) parameters of CJ-023,423 on day 21 were analysed in these experimental animals. The maximum plasma concentration (C_{max} at 0.5 h) was 2.8, 14 and 32 μgmL^{-1} after doses of CJ-023,423 of 29, 57 and 96 mgkg^{-1} twice daily, respectively. This confirmed that exposure to CJ-023, 423 increased in a dose-dependent manner.

To investigate the extent of involvement of EP$_4$ receptor, the effects of CJ-023,423 at 96 mgkg^{-1} twice daily on paw swelling, sialic acid, synovial inflammation and bone destruction were compared with those of rofecoxib at 5 mgkg^{-1} twice daily. There were no significant differences between these two

groups in contralateral paw swelling ($P=0.30$, unpaired Student's t-test). However, there were significant differences between the groups on ipsilateral paw swelling ($P=0.0004$, unpaired Student's t-test), sialic acid ($P=0.004$, unpaired Student's t-test), A/G ratio ($P=0.03$, unpaired Student's t-test), synovial inflammation ($P=0.01$, rank sum test) and bone destruction ($P=0.001$, rank sum test). Thus, CJ-023,423 had similar efficacy to rofecoxib in reducing oedema on the contralateral paw, and had weaker inhibitory effects than rofecoxib in reducing ipsilateral paw swelling, sialic acid, synovial inflammation and bone destruction.

Recently, it was reported that GW6273689X, an EP$_4$ antagonist, suppressed PGE$_2$-induced vasorelaxation of the saphenous vein in piglets (Wilson & Giles 2005; Wilson et al 2006). It is possible that the EP$_4$ receptor plays an important role in PGE$_2$-elicited plasma extravasation, mediated via

Table 2 Inhibitory effects of CJ-023,423 and rofecoxib on inflammatory biomarkers (serum sialic acid and albumin/globulin ratio) in rats with adjuvant-induced arthritis. Polyarthritis was induced by the injection of the adjuvant into the right hind paw. Oral administration of CJ-023,423 (29, 57 or 96 mg kg^{-1} twice daily), rofecoxib (0.5, 1.5 or 5 mg kg^{-1} twice daily) or vehicle was started on day 0 and continued until day 21. Blood was collected on day 22

Group	Sialic acid (mg mL^{-1})	Albumin/globulin ratio
Disease control	$1.86 \pm 0.04^{\dagger}$	$2.35 \pm 0.04^{\dagger}$
CJ-023,423 29 mg kg^{-1}	1.77 ± 0.04	2.54 ± 0.04**
CJ-023,423 57 mg kg^{-1}	1.65 ± 0.02**	2.55 ± 0.03**
CJ-023,423 96 mg kg^{-1}	1.64 ± 0.03**	2.70 ± 0.04**
Rofecoxib 0.5 mg kg^{-1}	1.78 ± 0.03	2.53 ± 0.03*
Rofecoxib 1.5 mg kg^{-1}	1.60 ± 0.04**	2.71 ± 0.07**
Rofecoxib 5 mg kg^{-1}	1.36 ± 0.08**	2.83 ± 0.04**
Normal control	0.90 ± 0.02	3.04 ± 0.03

Data are mean $\pm$ s.e.m. for eight rats. *$P < 0.05$, **$P < 0.01$ vs disease-control group (one-way analysis of variance followed by Dunnett's test); $^{\dagger}P < 0.001$ vs normal-control group (Student's unpaired t-test).
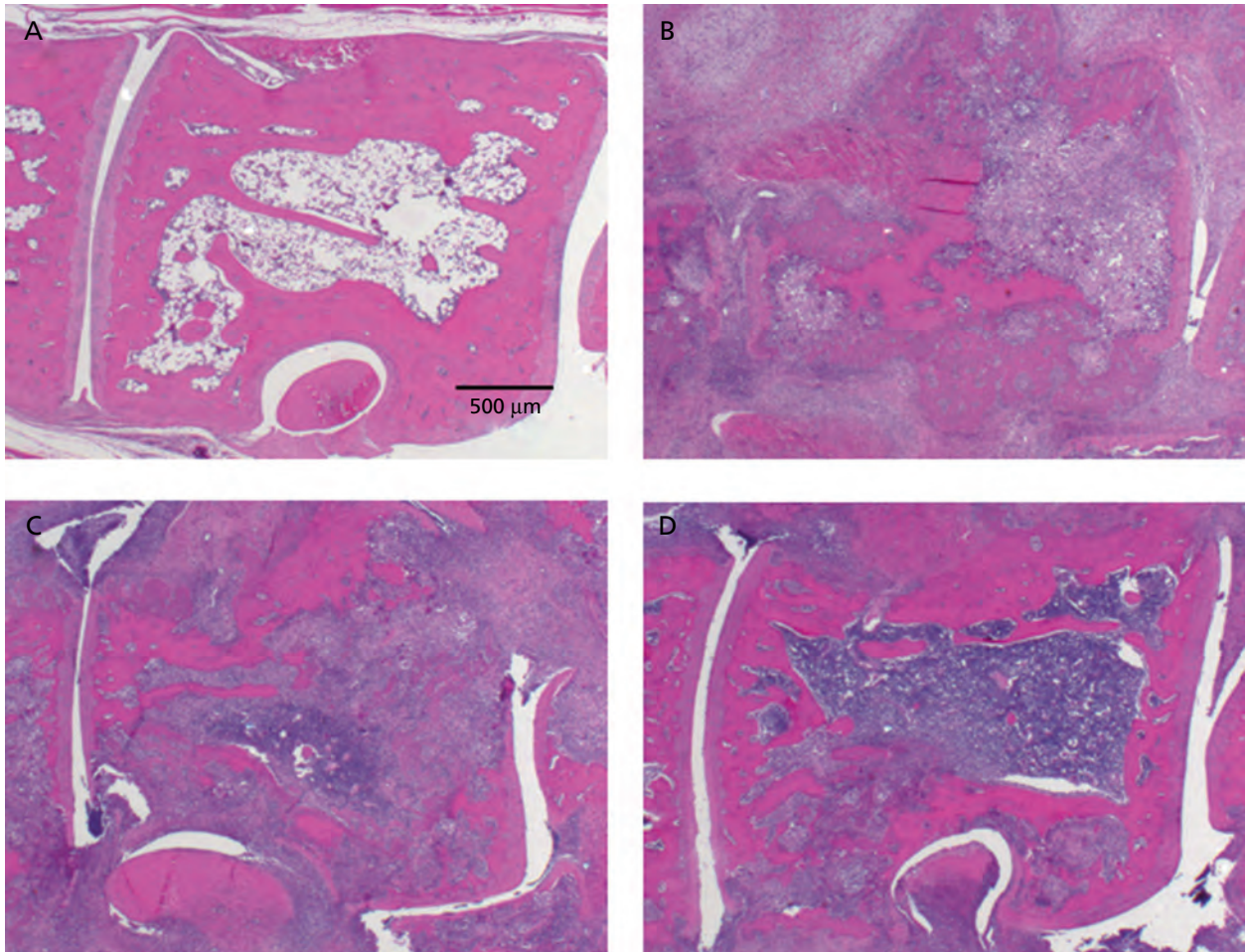

Figure 2 Low-magnification photomicrographs (H&E stain) of representative tarsal joint sections from rats with adjuvant-induced arthritis: (A) normal-control rat; (B) disease-control rat; (C) rat treated with CJ-023,423 96 mg kg^{-1} twice daily; (D) rat treated with rofecoxib 5 mg kg^{-1} twice daily. In the disease-control rat (B), the bone and joint tissues have been severely destroyed by inflammation. In the drug-treated rats (C and D), the severity of the lesions has been reduced compared with the disease-control rat. Higher-magnification images from A and B are shown in Figure 3. The length of the bar is 500 μm.
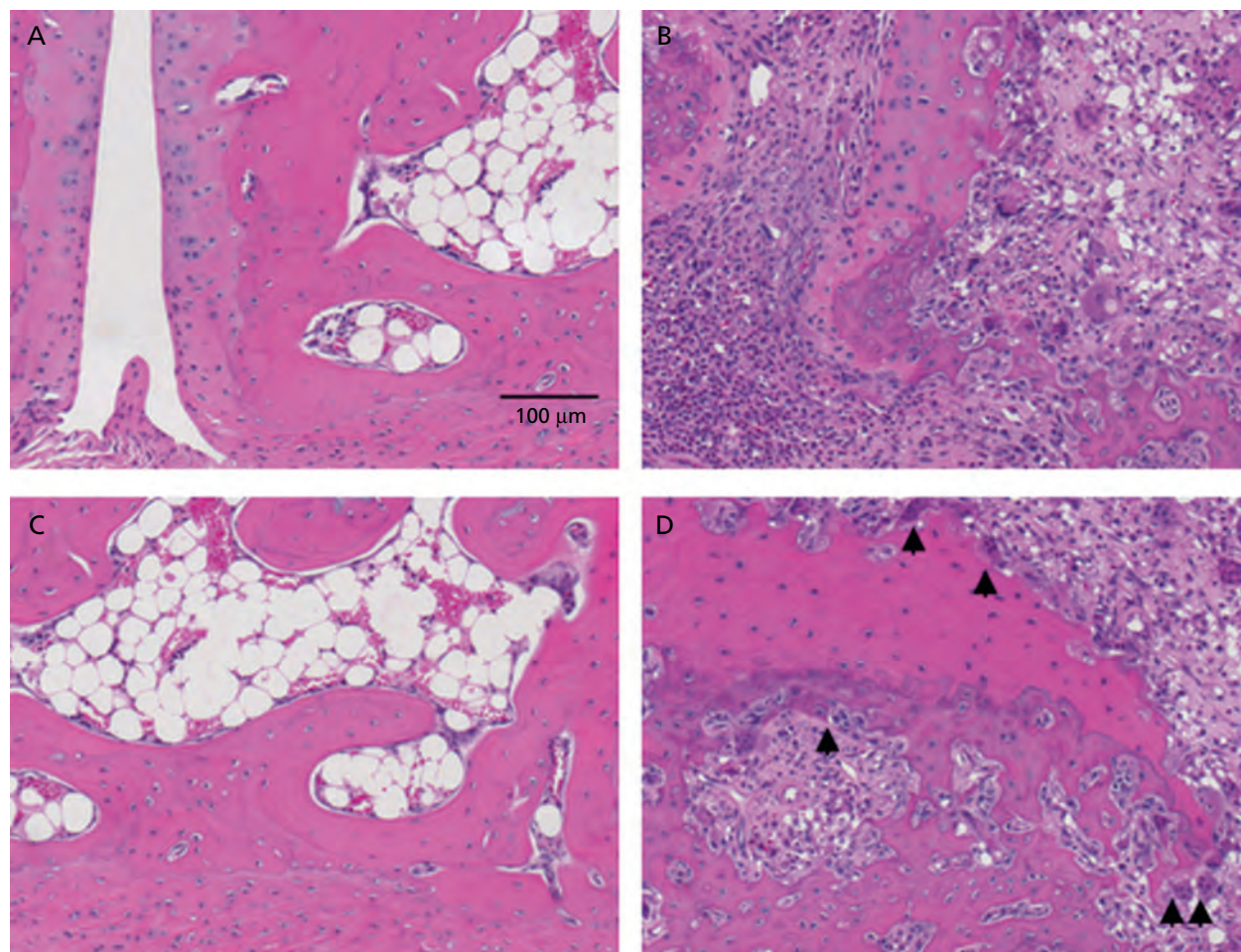
Figure 3 High-magnification photomicrographs (H&E stain) of representative tarsal joint sections from rats with adjuvant-induced arthritis: A and C are higher magnifications of Figure 2A; B and D are higher magnifications of Figure 2B. In the disease-control rat, the articular cartilage is eroded and there are severe inflammatory reactions in the joint cavity (B) the cortical bone is also eroded and newly formed bone trabeculae are seen adjacent to the existing cortical bone (D). Activated osteoclasts are observed on the bone surfaces (D, shown with arrows). The length of the bar is 100 μm.

Table 3 Resolution of synovial inflammation and bone destruction by CJ-023,423 and rofecoxib as determined by histopathological assessment of tarsal joint in rats with adjuvant-induced arthritis. Rats were given CJ-023,423 (29, 57 and 96 mg kg^{-1}), rofecoxib (0.5, 1.5 and 5 mg kg^{-1}) or vehicle twice daily for 22 days, starting on the day of adjuvant injection. The semiquantitative grading criteria used to evaluate synovial inflammation and bone destruction of the tarsal joints are shown in Table 1

Group	Inflammation score		Bone erosion score	
	Ipsilateral paw	Contralateral paw	Ipsilateral paw	Contralateral paw
Disease control	4.0 (4.0/4.0)[†]	4.0 (4.0/4.0)[†]	5.0 (5.0/5.0)[†]	5.0 (5.0/5.0)[†]
CJ-023,423 29 mg kg^{-1}	3.5 (3.0/4.0)	3.0 (3.0/3.0)**	4.5 (4.0/5.0)	4.0 (4.0/5.0)
CJ-023,423 57 mg kg^{-1}	3.0 (2.8/3.0)***	3.0 (3.0/3.0)**	4.0 (4.0/4.3)*	4.0 (4.0/5.0)
CJ-023,423 96 mg kg^{-1}	3.0 (3.0/3.0)**	3.0 (3.0/3.0)**	4.0 (4.0/4.3)*	4.0 (4.0/5.0)
Rofecoxib 0.5 mg kg^{-1}	3.0 (3.0/4.0)	3.0 (3.0/4.0)	4.5 (3.8/5.0)	4.0 (3.8/4.3)
Rofecoxib 1.5 mg kg^{-1}	2.5 (2.0/3.0)**	2.5 (2.0/3.0)***	3.0 (3.0/3.3)**	3.0 (3.0/3.0)***
Rofecoxib 5 mg kg^{-1}	2.0 (2.0/2.0)***	2.5 (2.0/3.0)***	3.0 (2.8/3.0)***	3.0 (2.0/3.0)***
Normal control	0.0 (0.0/0.0)	0.0 (0.0/0.0)	0.0 (0.0/0.0)	0.0 (0.0/0.0)

Data are median (1st/3rd quartiles); eight animals per group. *$P < 0.05$; **$P < 0.01$, ***$P < 0.001$ vs disease-control group (Kruskal–Wallis test followed by Dunn's multiple comparison test). [†]$P < 0.001$ vs normal-control group (rank sum test).

vasorelaxation, in chronic inflammatory reactions, leading to oedema.

It has been reported that COX-2 inhibitors suppress pro-inflammatory lipid mediators such as PGI$_2$ and PGF (Basu 2006; Pulichino et al 2006) in addition to PGE$_2$. It has also been reported that PGI$_2$–IP signalling and PGE$_2$–EP$_2$/EP$_4$ signalling mediated joint inflammation and bone destruction in a mouse CIA model (Honda et al 2006). CJ-023,423 may have had lower efficacy than rofecoxib in reducing synovial inflammation and bone destruction because efficacy of the latter also involves other lipid mediators produced by COX-2 in addition to PGE2 or other EP receptors in addition to the EP4 receptor. Selective receptor antagonists are needed to clarify these contributions in the AIA model.

Another reason for the lower efficacy of CJ-023,423 compared with rofecoxib may be a reflection of the short-lasting PK profile of CJ-023, 423. CJ-023,423 has a plasma half-life of 2.0 h (Nakao et al 2007) compared with 7.4 h for rofecoxib (Harirforoosh et al 2006) in rats. An EP$_4$ antagonist with a long duration of action would be useful to clarify the effects of EP$_4$ blockade on chronic inflammation and bone destruction in AIA rats.

Conclusions

In this study, CJ-023,423, an EP$_4$ antagonist, significantly suppressed paw swelling, synovial inflammation, inflammatory biomarkers and bone destruction in AIA rats. The inhibitory effect of CJ-023,423 on paw swelling was comparable to that of the COX-2 inhibitor rofecoxib. These results suggest that PGE$_2$ signalling via the EP$_4$ receptor is involved in the development of chronic inflammation, including bone destruction, and with particularly high sensitivity for oedema, in AIA rats. The involvement of EP$_4$ receptors in the pathogenesis of chronic inflammation and bone destruction in AIA rats is clarified with CJ-023,423. The relative selectivity of this compound for the EP$_4$ receptor makes it a useful tool to investigate the contribution of the EP$_4$ receptor in disease aetiology in-vivo.

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