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Effects of a novel non-steroidal anti-inflammatory drug (M-5011) on bone metabolism in rats with collagen-induced arthritis

Naofumi Murakami *, Hiroshi Takase, Takako Saito, Kazumi Iwata, Hiroaki Miura, Tomohiro Naruse

Research and Development Laboratories, Maruho, 1-8-23 Oyodo Naka, Kita-ku, Osaka 531-0076, Japan

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Abstract

The effects of a novel non-steroidal anti-inflammatory drug (NSAID), *d*-2-[4-(3-methyl-2-thienyl)phenyl]propionic acid: M-5011, and other NSAIDs (indomethacin, zaltoprofen and tiaprofenic acid) on bone metabolism in Dark Agouti (DA) strain rats with collagen-induced arthritis were evaluated. M-5011 (1.5 and 4.5 mg/kg) and other NSAIDs (1.5 mg/kg) were administered orally once a day from day 14 to day 35 after collagen immunization. In arthritic rats, paw volume and serum levels of anti-type II collagen antibody were increased on day 21 compared to those in non-immunized rats. M-5011 (4.5 mg/kg), indomethacin and zaltoprofen tended to prevent this increase in paw volume. Elevated urinary pyridinoline and deoxypyridinoline levels were found on days 28 and 35 in arthritic rats. M-5011 (4.5 mg/kg) also tended to prevent the increase in urinary pyridinoline level on day 28, but none of the other NSAIDs affected urinary deoxypyridinoline levels. Bone mineral densities in the hindpaw and vertebrae were also decreased in arthritic rats. M-5011 and tiaprofenic acid prevented this decrease in vertebral bone mineral density. These findings indicate that M-5011 partially inhibits the generalized bone loss accompanying the development of collagen-induced arthritis in rats.

Keywords: Collagen arthritis; Pyridinoline; Bone mineral density; NSAIDs (non-steroidal anti-inflammatory drugs)

1. Introduction

Collagen-induced arthritis in mice (Courtenay et al., 1980) and rats (Trentham et al., 1977) is a useful model to study the pathogenesis of rheumatoid arthritis. In this model, arthritis is usually induced by immunization of native type collagen in rats such as Dark Agouti (DA) rats, the most susceptible strain (Griffiths et al., 1981). This arthritis is associated with a high level of both humoral and cellular immunity to type II collagen (Stuart et al., 1982). Radiographic examination reveals bone matrix resorption (predominantly subchondral bone), formation of osteophytes at the joint margin and swelling in the tibiotarsal joint (Smith and Sly, 1996) in animals with this type of arthritis. However, it is not known whether generalized bone loss occurs in rats with collagen-induced arthritis. Urinary pyridinoline and deoxypyridinoline are markers of bone metabolism, reflecting bone and cartilage turnover in patients with arthritis (Seibel et al., 1989). Therefore, measurement of levels of these markers is quite useful for determining both the pathological changes of bone and the efficacy of drug therapy for rheumatoid arthritis.

Nonsteroidal anti-inflammatory drugs (NSAIDs) such as indomethacin are widely used for the treatment of rheumatoid arthritis (Jean-Pierre et al., 1994). However, the use of NSAIDs is limited by their undesirable gastrointestinal ulcerogenetic effects, which are the result of inhibition of prostaglandin biosynthesis (Whittle, 1981). Indomethacin at a dose of 5.0 mg/kg induced pronounced weight loss in rats with collagen-induced arthritis (Kamada et al., 1997). Thus, significant effort has been directed toward the development of a potent NSAID with low ulcerogenicity. We recently produced a novel acidic NSAID, *d*-2-[4-(3-methyl-2-thienyl)phenyl]propionic acid (M-5011), which has potent anti-inflammatory, analgesic and anti-pyretic activities with low gastrointestinal ulcerogenic activity (Murakami et al., 1996; Kido et al., 1998). A previous study showed that M-5011 down-regulates interleukin-6 production at both the protein and mRNA levels in vitro (Kanemoto et al., 1998). The effects of M-5011 on

* Corresponding author. Tel.: +81-6-4530063; fax: +81-6-4530076.

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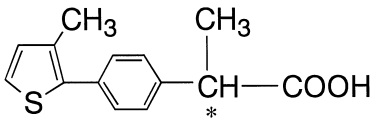

Fig. 1. Structure of *d*-2-[4-(3-methyl-2-thienyl)propionic acid (M-5011). *: Asymmetrical carbon atom.

interleukin-6 production, but not prostaglandin E_2 production, are significantly more potent than those of indomethacin. In addition, daily administration of M-5011 reduced hindpaw swelling in rats with collagen-induced arthritis in preliminary experiments. These findings suggest that the mechanisms by which M-5011 inhibits this arthritis may differ from those of indomethacin.

The purpose of the present study was to examine systemic changes in bone mineral density by dual-energy X-ray absorptiometry and measurement of urinary markers of bone metabolism (pyridinoline and deoxypyridinoline) in rats with collagen-induced arthritis. In addition, we evaluated the effects of NSAIDs, including M-5011, on these changes in bone.

2. Materials and methods

2.1. Animals and materials

Experiments were performed on female DA strain rats (8 weeks of age at purchase; Shizuoka Laboratory Center, Shizuoka, Japan) that were allowed to adapt to their environment for 1 week. All animals had free access to food and water and were kept in a room maintained at constant temperature (23 ± 2°C) and relative humidity (55 ± 10%) on a 12-h light/dark cycle (lights on 0700 h).

The chemical structure of *d*-2-[4-(3-methyl-2-thienyl)phenyl]propionic acid, M-5011 (Maruho, Osaka, Japan), is shown in Fig. 1. Indomethacin was purchased from Sigma (St. Louis, MO, USA). Zaltoprofen and tiaprofenic acid were prepared from Soleton® (Nippon Chemiphar, Tokyo, Japan) and Surgam® (Nippon Roussel,

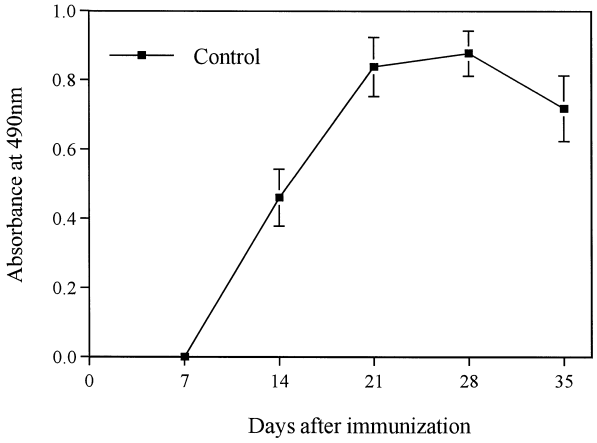

Fig. 3. Changes in serum type II collagen antibody level in rats with arthritis. Each value represents the mean ± S.E. ($n = 7$–8).

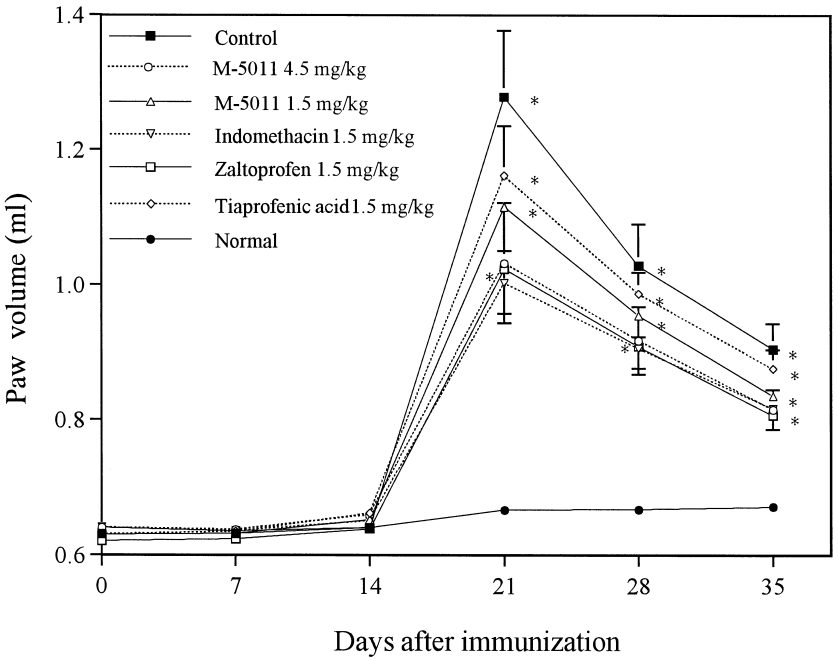

Fig. 2. Effects of M-5011, indomethacin, zaltoprofen and tiaprofenic acid on paw volume in rats with type II collagen-induced arthritis. Each group was given drug or vehicle (control) orally once a day from day 14 to day 34 after immunization. A normal group that was not treated with either type II collagen or vehicle was also prepared. Paw volume was measured on days 0, 7, 14, 21, 28 and 35. Each value represents the mean ± S.E. The number of animals per group was eight, except for the control group ($n = 7$) on both days 28 and 35. *Significantly different from normal group (Kruskal–Wallis test significant at $P < 0.05$; significantly different from normal group as determined by Dunn's multiple comparison method with experiment-wise error rate controlled at $\alpha = 0.05$).

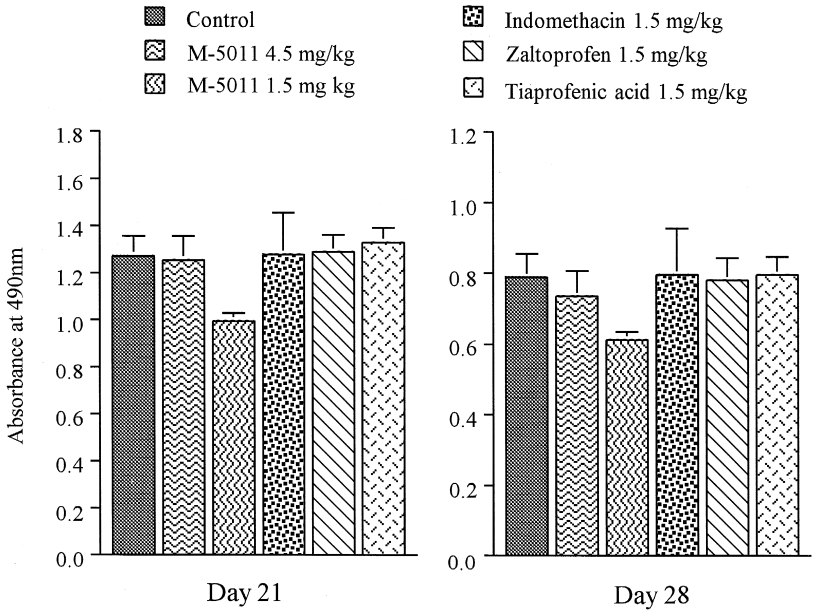

Fig. 4. Effects of M-5011, indomethacin, zaltoprofen and tiaprofenic acid on serum type II collagen antibody level in rats with arthritis. Serum type II collagen antibody levels were measured on days 21 and 28. Each value represents the mean ± S.E. ($n = 7–8$).

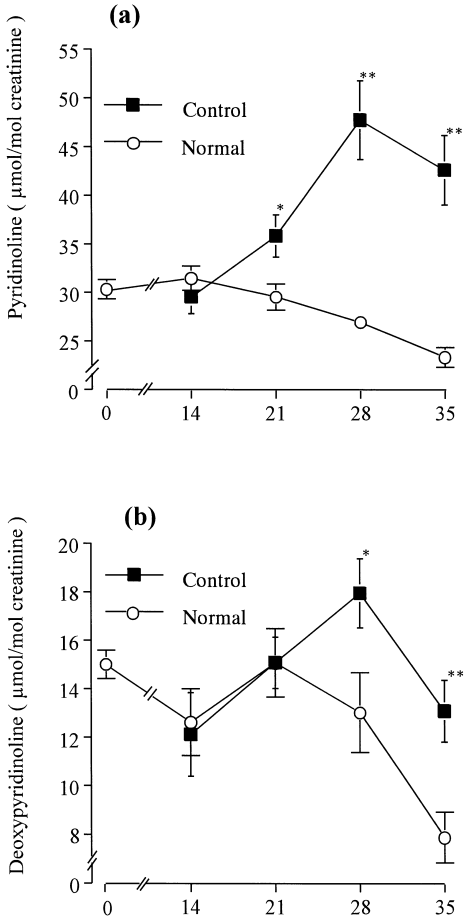

Fig. 5. Urinary pyridinoline (a) and deoxypiridinoline (b) levels for rats with type II collagen-induced arthritis. Each value represents the mean ± S.E. ($n = 7–8$). Significantly different from normal group at each time point, $* P < 0.05$ and $** P < 0.01$, Aspin–Welch test.

Tokyo, Japan), respectively. The purity of both of the latter NSAIDs, as assessed by high-performance liquid chromatography (HPLC), was greater than 99.5%. M-5011 (4.5 mg/kg) and reference drugs (1.5 mg/kg) were suspended in 0.5% carboxymethylcellulose sodium (CMC: Wako Pure Chemical, Osaka, Japan). Lower concentrations of M-5011 (1.5 mg/kg) were prepared by serial dilution in vehicle (CMC) whenever applicable.

2.2. Experimental protocol

Rats aged 9 or 10 weeks were randomly assigned to various groups according to the experimental design. Each group was orally administered drug or vehicle (control) once a day from day 14 to day 35 after collagen immunization. The normal group was not treated with either type II collagen or vehicle. To clarify the radiographic changes and changes in bone mineral density during the development of collagen-induced arthritis, eight rats in the normal group were killed on day 0 or day 21 ($n = 4$, respectively). Sixteen rats in the control group were also killed to examine the changes in bone mineral density on day 7, day 14, day 21 and day 28 ($n = 4$, respectively). In other rats in each group ($n = 8$), paw volume and serum type II collagen antibody level were measured on days 0, 7, 14, 21, 28 and 35. Urinary pyridinoline and urinary de-

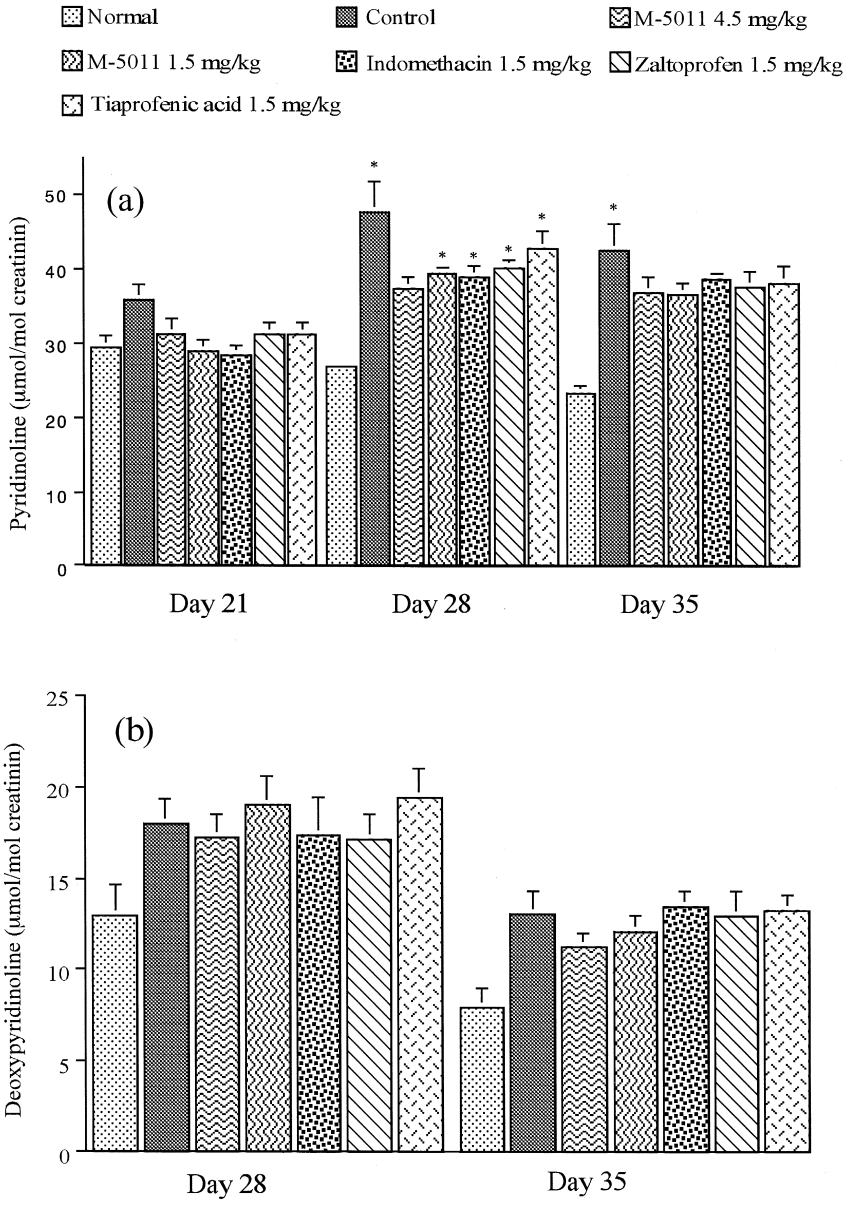

Fig. 6. Effects of M-5011, indomethacin, zaltoprofen and tiaprofenic acid on urinary pyridinoline (a) and deoxypiridinoline (b) levels for rats with type II collagen-induced arthritis. Urinary pyridinoline was measured on days 21, 28 and 35. Urinary deoxypiridinoline was measured on days 28 and 35. Each value represents the mean ± S.E. ($n = 7$–8). *Significantly different from normal group (Kruskal–Wallis test significant at $P < 0.05$; significantly different from normal group as determined by Dunn's multiple comparison method with experiment-wise error rate controlled at $\alpha = 0.05$).

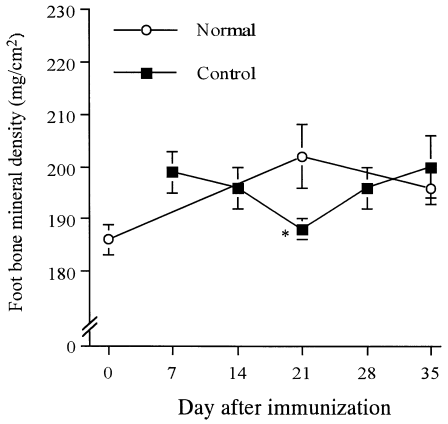

Fig. 7. Changes in hindpaw bone mineral density in rats with type II collagen-induced arthritis. Each value represents the mean ± S.E. The number of animals per group was four, except for the normal group on day 35 ($n = 8$) and the control group on day 35 ($n = 7$). Significantly different from normal group at each time point, $* P < 0.05$, Aspin–Welch test.

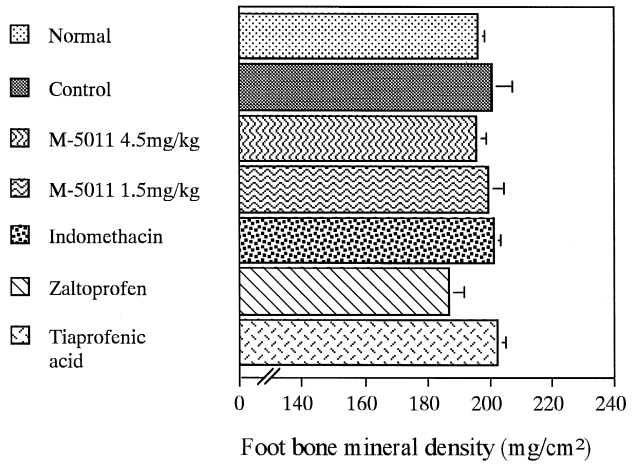

Fig. 9. Effects of M-5011, indomethacin, zaltoprofen and tiaprofenic acid on hindpaw bone mineral density in rats with type II collagen-induced arthritis. Hindpaw bone mineral density was measured on day 35. Each value represents the mean ± S.E. ($n = 7$–8).

oxypyridinoline were measured on days 14, 21, 28 and 35. In addition, other rats in each group were killed to determine bone mineral density on day 35.

2.3. Induction of type II collagen arthritis and inflammatory parameters

Bovine type II collagen from arthritic cartilage (Cosmo Bio, Tokyo, Japan) dissolved in 0.01 M acetic acid (2 mg/ml) was diluted 1:11 with saline, emulsified in an equal volume of Freund's incomplete adjuvant (Nacarai Tesque, Kyoto, Japan), and kept cold in an ice bath. Collagen arthritis was induced by intradermal injection of 0.5 ml (50 μg/body) cold emulsion at four sites on the back of rats in all groups except the normal group on day 0.

The volume of both hind paws was measured with a water plethysmometer (Model TK101, Unicom, Chiba, Japan), and the mean volume (ml) of the two was calculated as a parameter of inflammation.

On days 7, 14, 21, 28 and 35 after collagen injection, blood was routinely collected by retroorbital bleeding under ether anesthesia, using a capillary tube, and centrifuged. Samples were stored at −80°C until assayed. Serum levels of antibodies to type II collagen were measured with an enzyme-linked immunosorbent assay (ELISA) system (Collagen Gijyustu Kenshukai, Sendai, Japan).

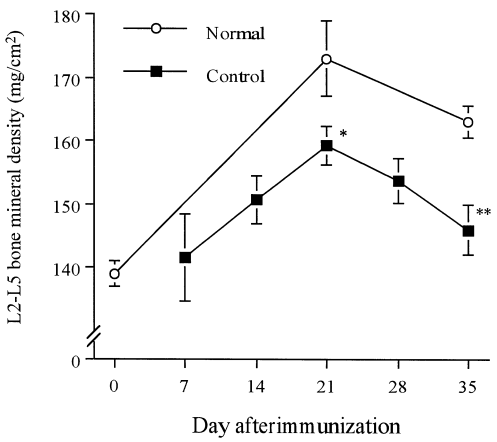

Fig. 8. Changes in L2–L5 lumbar vertebral bone mineral density in rats with type II collagen-induced arthritis. Each value represents the mean ± S.E. The number of animals per group was four, except for the normal group on day 35 ($n = 8$) and the control group on day 35 ($n = 7$). Significantly different from normal group at each time point, $* P < 0.05$ and $** P < 0.01$, Aspin–Welch test.

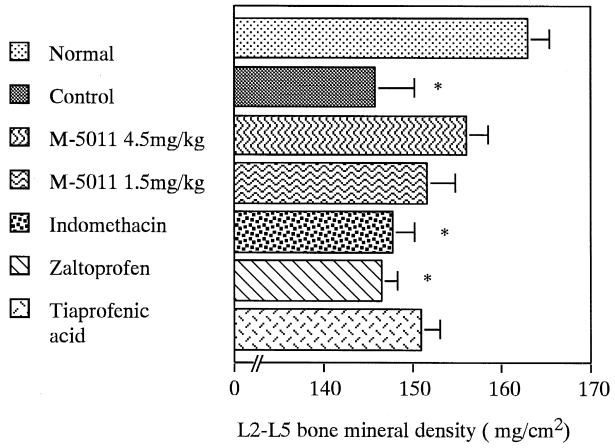

Fig. 10. Effects of M-5011, indomethacin, zaltoprofen and tiaprofenic acid on L2–L5 lumbar vertebral bone mineral density in rats with type II collagen-induced arthritis. Lumbar vertebral mineral density was measured on day 35. Each value represents the mean ± S.E. ($n = 7$–8). *Significantly different from normal group (ANOVA significant at $P < 0.05$; significantly different from normal group as determined by the parametric Bonferroni multiple comparison method with experiment-wise error rate controlled at $\alpha = 0.05$).

2.4. Urinary pyridinoline and deoxypyridinoline measurement

Urine was collected over a 24-h period and centrifuged ($1000 \times g$ for 15 min). Samples were stored at -80°C until measurement. Pyridinoline and deoxypyridinoline were measured by fluorometric assay using HPLC, after acid hydrolysis of the urine. Urinary excretion of pyridinoline and deoxypyridinoline was corrected for the urinary creatinine level (μmol/mol creatinine). Urinary creatinine levels were determined using the Wako Creatinine-Test (Wako) by the Jaffe rate technique with alkaline picrate.

2.5. Bone densitometry and radiography

The hindlimbs and vertebrae were dissected bilaterally, and fixed with phosphate-buffered 10% formaldehyde. The bone mineral density of the hindpaws and vertebrae (L2 to L5) was measured bilaterally by dual-energy X-ray absorp-

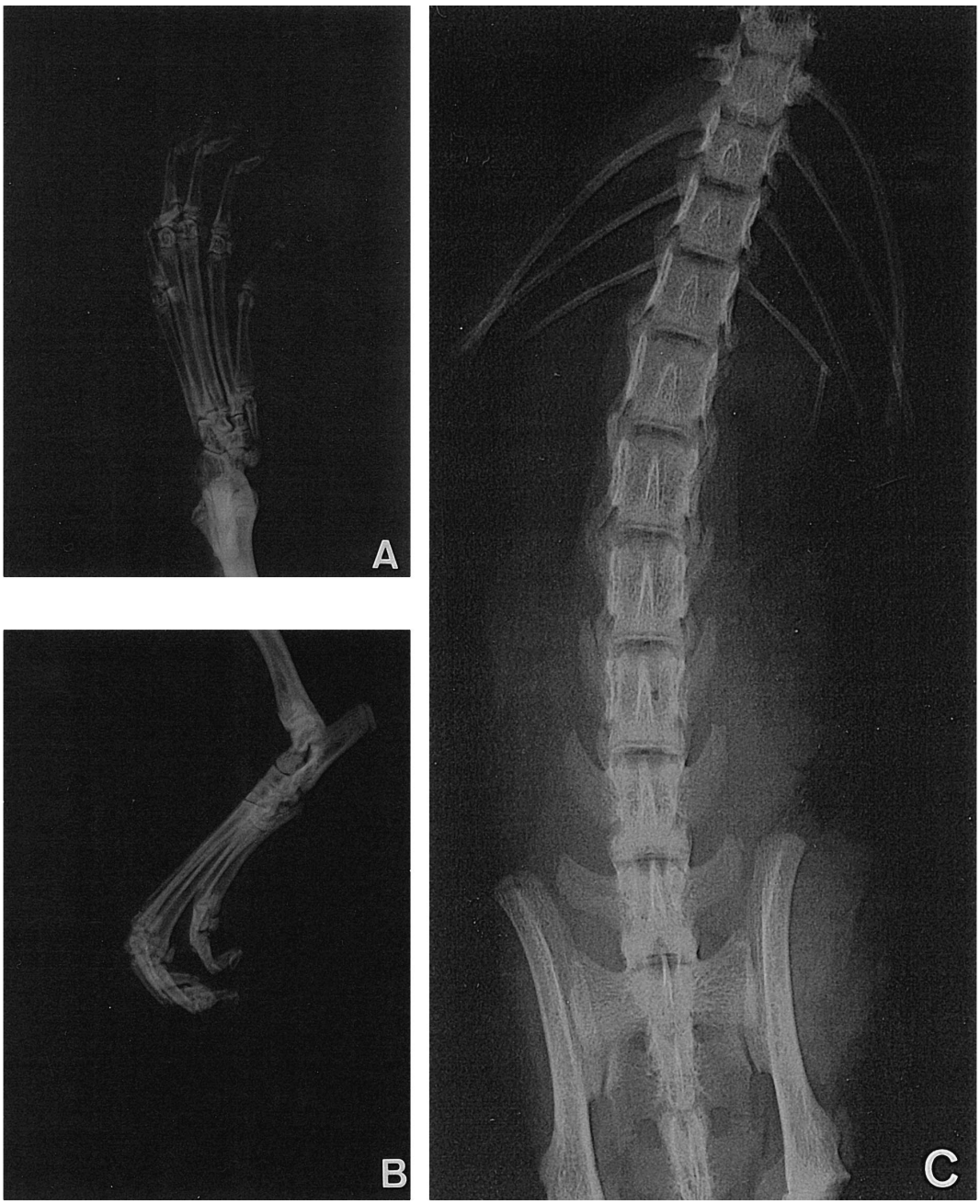

Fig. 11. Soft X-ray radiographs of the hindpaws and lumber vertebrae in rats. Radiographs A, B and C are of normal rats and radiographs D, E and F are of rats with type II collagen-induced arthritis on day 35. Radiographs D and E show typical arthritic lesions. Arrows indicate new bone growth (d) and bone destruction (e).

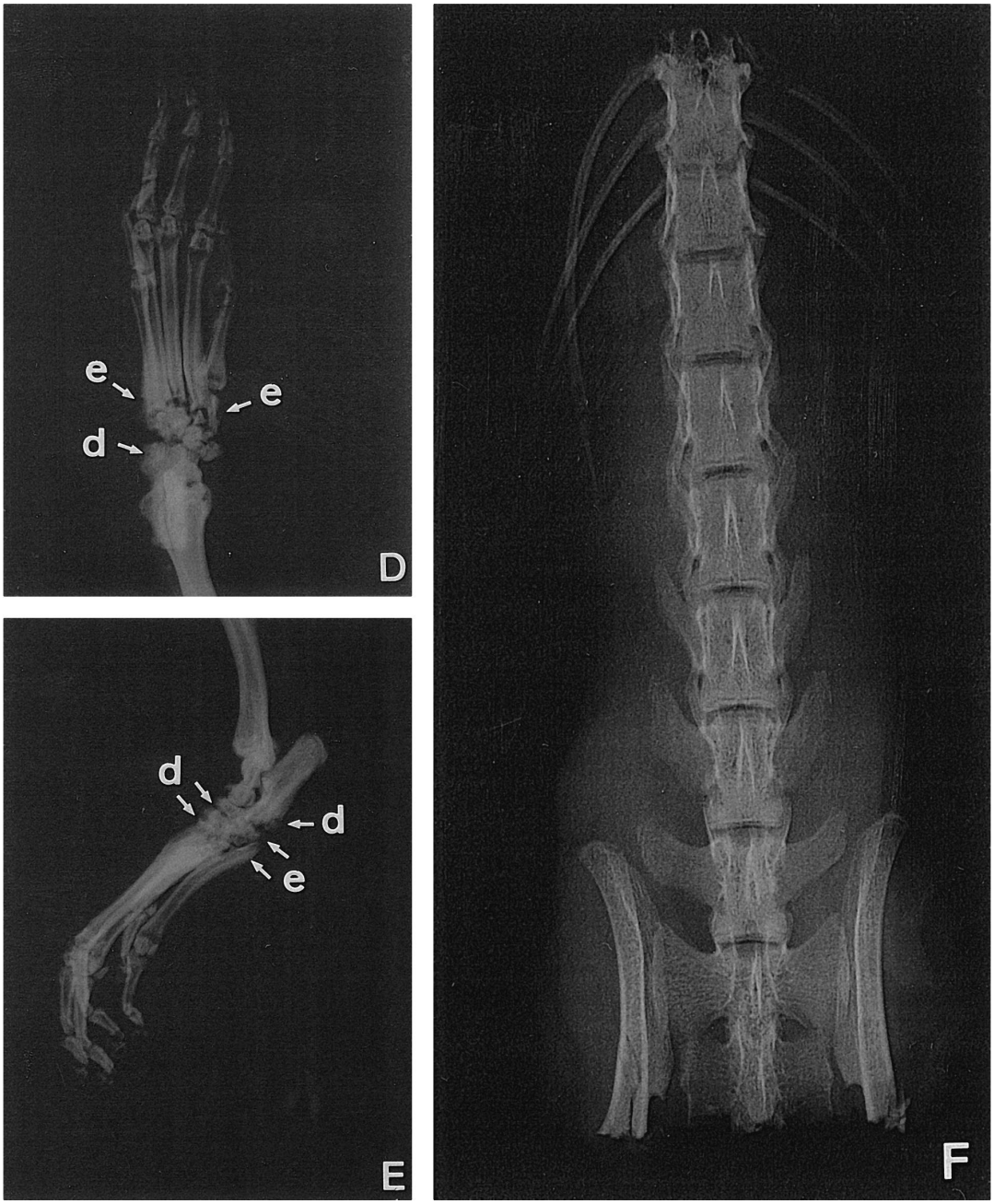

Fig. 11 (continued).

tiometry (QDR-1000, Hologic, MA, USA). Soft radiography of the hindpaws and vertebrae was performed using a soft X-ray apparatus (25 kV, 2.5 mA, 60 s; CSM-2 Special Model, Softex, Tokyo, Japan)

2.6. Statistical analysis

Results are expressed as means ± S.E. Prior to group comparisons, Bartlett's test was used to determine whether variances were homogeneous or not. Data with homogeneous variance were analyzed by one-way analysis of variance (ANOVA). If significant ($P \leq 0.05$) differences were observed, differences between groups were tested by the parametric Bonferroni multiple comparison method with experiment-wise error rate controlled at $\alpha = 0.05$. Data with non-homogeneous variance (i.e., when Bartlett's test was significant, $P \leq 0.05$) were analyzed by Kruskal–Wallis nonparametric ANOVA. If significant ($P \leq 0.05$) differences were observed, differences between groups were tested using Dunn's distribution-free method. The *F*-test of variance followed by Student's *t*-test was initially used to compare values between normal and control rats. If variances were not homogeneous, the Aspin–

Welch *t*-test was used. When only two means values were compared, the level of significance was set at $P \leq 0.05$.

3. Results

3.1. Paw volume and serum type II collagen antibody levels

Swelling of the ankle joint developed on day 14 through day 17 after immunization with collagen. Hindpaw volume was maximal at 1.28 ± 0.10 ml on day 21, and then decreased gradually (Fig. 2). None of the test drugs significantly reduced paw volume compared with that of the control rats. However, M-5011 (4.5 mg/kg), indomethacin and zaltoprofen each tended to reduce paw volume.

Serum levels of anti-type II collagen antibody were not detectable on day 7, peaked (absorbance at 490 nm: 0.877 ± 0.08) on day 21, and then decreased slightly by day 35 (Fig. 3). None of the test drugs affected serum type II collagen antibody concentration on either day 21 or day 28 (Fig. 4).

3.2. Urinary pyridinoline and deoxypyridinoline

Urinary pyridinoline levels were higher in the arthritic rats ($P < 0.05$) than in the normal rats (26.94 ± 0.64 μM/creatinine) on day 21, peaked (47.75 ± 4.04 μM/creatinine) on day 28, and then decreased slightly (42.6 ± 3.57 μM/creatinine) by day 35 (Fig. 5a). Urinary deoxypyridinoline levels in the arthritic groups were significantly higher than in the normal group on days 28 and 35 ($P < 0.05$ and $P < 0.01$, respectively), at 17.95 ± 1.44 and 13.08 ± 1.27 μM/creatinine, respectively (Fig. 5b).

Levels of pyridinoline were significantly higher in all test drug groups, except that of M-5011 (4.5 mg/kg), than in the normal group ($P < 0.05$, Fig. 6a). Only M-5011 (4.5 mg/kg) tended to decrease pyridinoline level on day 28. None of the test drugs affected deoxypyridinoline level on either day 28 or day 35 (Fig. 6b).

3.3. Bone mineral density

Bone mineral density in the hindpaw of the arthritic rats was significantly lower on day 21 (188.23 ± 2.16 mg/cm^2) than in the normal rats ($P < 0.05$), and then plateaued on day 35 (202.40 ± 5.92 mg/cm^2) (Fig. 7). In the arthritic rats, bone mineral density in the L2–L5 vertebrae was consistently lower (Fig. 8) than in the normal rats throughout the experimental period. On days 21 and 35, L2–L5 bone mineral density was significantly lower in the arthritic group than in the normal group ($P < 0.05$ and $P < 0.01$, respectively), at 172.55 ± 6.08 mg/cm^2 and 162.89 ± 2.53 mg/cm^2, respectively.

None of the test drugs affected hindpaw bone mineral density on day 35 (Fig. 9). However, on day 35 L2–L5 vertebral bone mineral density in rats treated with M-5011 (1.5 and 4.5 mg/kg) and tiaprofenic acid (Fig. 10) was not significantly lower than in the normal group. None of the other test drugs affected the decrease in vertebral bone mineral density.

3.4. Radiographic changes in hindpaws and vertebrae

Radiographic examination of both hindpaws of the arthritic rats on day 35 revealed osteophyte formation, narrowing of the joint spaces, new bone growth (d) and subsequent bone and cartilage destruction (e) in the tibiotarsal joint (Fig. 11D and E), compared with findings for normal rats (Fig. 11A and B). However, no pathological changes were found in immunized rat vertebrae (Fig. 11C and F).

4. Discussion

Recent studies have confirmed that adjuvant-induced arthritis is associated with both a decrease in bone formation and an increase in bone resorption due to an increased production of prostaglandins (Bonnet et al., 1993; Segawa et al., 1995; Aota et al., 1996). Adjuvant-induced arthritis is an acute, severe disease characterized by the systemic appearance of granulomas, but not polyarthritis (Pearson and Wood, 1959). Collagen-induced arthritis accompanied by chronic relapsing joint disease (Chang et al., 1980; Vingsbo et al., 1995) is a suitable model of human rheumatoid arthritis.

In the present study, treatment with M-5011 (4.5 mg/kg), indomethacin (1.5 mg/kg) and zaltoprofen (1.5 mg/kg) tended to reduce paw swelling on days 14–35 after type II collagen immunization without affecting serum levels of anti-type II collagen antibody. These findings clearly indicate that the development of arthritis is accompanied by striking changes in the metabolic turnover of collagen in bone and tendon. Urinary pyridinoline and deoxypyridinoline levels were significantly increased on days 21–28 and days 28–35, respectively. These peaks of urinary pyridinoline and deoxypyridinoline levels occurred later than the peak of paw swelling, indicating that these parameters did not correlate directly with acute polyarthritic inflammation. However, a local inflammatory process is likely to play a key role in the changes in urinary pyridinium cross-links in these animals. In contrast to a previous clinical report (Seibel et al., 1989) that treatment with NSAIDs had no effect on urinary cross-link excretion, in the present study daily treatment with M-5011 (4.5 mg/kg) or indomethacin (1.5 mg/kg) reduced the urinary pyridinoline level. Pyridinoline is the predominant cross-link in cartilage, and deoxypyridinoline is primarily located in the collagenous matrix of bone (Woolf, 1991). Although these two parameters are increased in rheumatoid arthritis, urinary pyridinoline is related to disease

activity, whereas urinary deoxypyridinoline reflects a generalized increase in bone turnover and loss.

At the peak of paw swelling in rats with collagen-induced arthritis, tibiotarsal and lumbar bone mineral density was significantly lower than in the normal rats. Tibiotarsal bone mineral density returned to control level by day 35, although vertebral bone mineral density continued to be lower than in controls. These findings suggest that the changes observed in mineral density in tibiotarsal bone reflect acute arthritic inflammation rather than chronic bone metabolism. The decrease in bone mineral density observed on days 21 and 35, which was significant only for vertebrae, suggested that the vertebrae are more sensitive than tibiotarsal bone to the increase in urinary pyridinium cross-links in rats with collagen-induced arthritis. Although none of the NSAIDs tested affected tibiotarsal bone mineral density, treatment with M-5011 and tiaprofenic acid tended to prevent the decrease in bone mineral density in vertebra at non-inflamed sites. Urinary pyridinoline and deoxypyridinoline levels are important markers of bone turnover in elderly women with vertebral osteoporosis (Delmas et al., 1991). Our findings therefore suggest that some NSAIDs, including M-5011, can help prevent the progression of generalized osteoporosis.

The mechanisms underlying the change in generalized bone metabolism in collagen-induced arthritis are still unknown. However, prostaglandins may be important regulators of bone and cartilage turnover. Prostaglandin E_2 in particular has both potent bone resorptive activity (Raisz et al., 1977; Tashijian et al., 1977) and bone formation activity in vivo (Takada et al., 1995; Ke et al., 1992; Takagi et al., 1993). Prostaglandins (mainly prostaglandin E_2 and prostaglandin I_2) thus have dual effects on the resorption and formation of bone. It has in fact been reported that the release of prostaglandin E_2 from chondrocyte cultures is markedly inhibited by tiaprofenic acid (Ghosh, 1993). We have already reported that M-5011 exhibits weaker inhibition of prostaglandin I_2 synthesis than does indomethacin, but more potent inhibition than does tiaprofenic acid or zaltoprofen (Murakami et al., 1996). However, these inhibitory potencies are not consistent with our findings for vertebral bone mineral density, which were indicative of generalized osteoporosis.

Elevated serum levels of cytokines including tumor necrosis factors, γ-interferon, and interleukin-2 and -6 have been found in patients with rheumatoid arthritis (Jones and Bhalla, 1993). Elevated levels of interleukin-1 and -6 were also observed in rats with collagen-induced arthritis as well as in patients with rheumatoid arthritis (Sugita et al., 1993). The relationships among cytokines, cyclooxygenase activation and prostaglandin E_2 production are very complex. Interleukin-1 β is a potent stimulator of cyclooxygenase in many different types of cells including synoviocytes. However, prostaglandins have negative feedback effects on interleukin-1 production. In fact, the inhibition by NSAIDs of cyclooxygenase activity has been shown to increase interleukin-1 production in a rat pleurisy model (Utsunomiya et al., 1994). In contrast to the effect of interleukin-1, an inhibitory effect of prostaglandins on interleukin-6 production has not been clearly demonstrated. Results obtained in vitro (Kanemoto et al., 1998) suggest that daily treatment with either indomethacin or M-5011 before type II collagen immunization may reduce serum interleukin-6 levels in rats with collagen-induced arthritis. Inhibitory effects of some NSAIDs on the production of interleukin-6 have also been demonstrated in rats with arthritis (Theisen-Popp et al., 1992; Leistein et al., 1990). Furthermore, levels of interleukin-1 and -6 in bone marrow supernatant increased prior to the onset of synovial proliferation in rats with collagen-induced arthritis (Fujimoto et al., 1992). The cells and mediators involved in this inflammatory process, such as mast cells and their products, monocytes and interleukin-1, may be responsible for increased bone resorption (Avoli, 1987). Further studies delineating the effect of these cytokines on collagen-induced arthritis in rats will be needed to clarify the mechanism of vertebral bone loss in this experimental model.

In conclusion, our findings suggest that M-5011 partially inhibits the generalized bone loss that accompanies the development of collagen-induced arthritis in rats, and that M-5011 may be effective in preventing the progression of generalized osteoporosis in rheumatoid arthritis.

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