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High bone turnover state under osteoporotic changes induces pain-like behaviors in mild osteoarthritis model mice

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Abstract

Introduction Our previous studies demonstrated that a high bone turnover state under osteoporotic changes decreased the threshold of skeletal pain. Recent studies reported that the incidence of joint pain due to osteoarthritis (OA) in postmenopausal women was higher than that in males even with the same radiographic OA grade. The aim of this study was to evaluate whether a high bone turnover state affects the induction of pain-like behaviors in mild OA model mice.

Method We established mild OA model mice with accompanying osteoporotic changes by monosodium iodoacetate injection after ovariectomy. We assessed pain-like behaviors by von Frey test and paw-flick test; histological changes in OA joints; the expression of Runx2, Osterix, Osteocalcin, and Rankl; bone micro-architecture by μCT and measured serum tartrate-resistant acid-phosphatase 5b levels in the model mice.

Results Pain-like behaviors in mice with OA and osteoporotic changes were significantly increased in comparison with those in OA mice without osteoporotic changes. The severity of histological OA changes did not differ significantly between the OA mice with and without osteoporotic changes. Bisphosphonate significantly improved pain-like behaviors accompanied with improvement in the high bone turnover state in the OA mice with osteoporosis, while it had no significant effect on pain-like behaviors in the OA mice without osteoporosis. In addition, the improvement was maintained for more than 4 weeks even after the discontinuation of bisphosphonate treatment

Conclusion These results indicated that a high bone turnover state under osteoporotic changes could affect the induction of pain-like behaviors in mild OA model mice.

Key words

osteoporosis, osteoarthritis, high bone turnover, pain, bisphosphonate

Introduction

Osteoporosis is characterized by a high bone turnover state with decreased bone mass and increased risk of fragility fracture in postmenopausal patients [1]. The number of patients with osteoporosis is estimated at 12,800,000 in Japan [2,3]. Osteoarthritis (OA) is characterized by pathologic features including joint space narrowing and osteophytosis, and represents a major public health issue associated with chronic skeletal pain in the elderly [4]. The number of patients with radiographic knee OA is estimated at 25,300,000 in Japan [3]. The proportion of patients with osteoporosis or OA increases with age, with the incidence of osteoporosis, in particular, increasing in postmenopausal women.

Interestingly, a previous study showed that in the patients with radiographic OA aged 50 years or older, the incidence of joint pain in females was higher than that in males. On the other hand, in patients younger than 50 years, the incidence of pain in males with radiographic knee OA was higher than that in females [5]. Another study demonstrated that the incidence of pain in female patients was higher than that in males even with the same radiographic OA grade as diagnosed using the Kellgren/Lawrence (K/L) grading system [6]. These results suggest that the postmenopausal condition in women aged 50 years or over involves an increase in joint pain due to OA, which might derive from a different mechanism from that in men.

With regard to the pathological relationship between osteoporosis and OA, there have been a number of studies on the underlying mechanisms [7,8], with most which focused on pathological changes in subchondral bone metabolism and inflammatory changes in joints, which were shown to affect the induction of pain [9-11].

We previously reported that a high bone turnover state under osteoporotic changes decreased the pain threshold in the limbs of ovariectomized (OVX) mice, which we used as a postmenopausal osteoporosis model [12-15] . We, therefore, hypothesized that a patient with osteoporosis would complain more strongly of joint pain due to OA under a high bone turnover state in comparison with a patient without osteoporosis, even one with the same radiographic OA grade.

In this study, we established model mice with mild OA with accompanying osteoporotic changes by OVX, and evaluated whether the decreased pain threshold due to a high bone turnover state induced pain-like behaviors in the model mice.

Materials and Methods

Animals

The present experiments were approved by the University Animal Care Committee of our institution (Approval number: 16-087) and were undertaken in accordance with the ethical guidelines of the National Institute of Health. Every effort was made to minimize animal suffering and the number of animals used. Experiments were conducted on 8-week-old female C57BL/6J mice weighing 20–25 g (Japan SLC, Hamamatsu, Japan). The mice were housed in a temperature-controlled room at 21 °C with a 12h light/dark cycle, and were given free access to food and water. The animals were anesthetized with pentobarbital (0.5ml/kg) and 5% isoflurane for experiments involving OVX and monosodium iodoacetate (MIA) joint injection. The animals were humanely sacrificed with an intraperitoneal injection of pentobarbital sodium (0.5 mg/kg) and inhalation of 5% isoflurane after completion of the experiments.

We used a total of 141 mice including controls (without OVX or MIA injection), osteoporosis mice (no MIA injection after OVX), OA mice (MIA injection without OVX) and OA with osteoporosis mice (MIA injection after OVX).

Osteoporosis and OA model mice

The 8-week-old mice were bilaterally ovariectomized to induce osteoporotic changes with a high bone turnover state as in our previous studies [12]. The sham operation was performed using the same surgical procedure as the OVX operation, apart from ovary removal. The OVX (n = 50) and sham (n = 51) surgeries were performed using a minimally invasive technique through a dorsal approach. The OVX mice showed a significant increase in bone resorption markers at 4 weeks, and a significant decrease in bone mineral density (BMD) and changes in micro-architecture according to micro-computed tomography (μCT) analysis of the femur at 8 weeks after surgery [12].

OA was induced by an injection of monosodium iodoacetate (MIA, Sigma-Aldrich, St. Louis, MO) into the left knee joint at 6 weeks after OVX (Supplementary Fig. 1). The doses of MIA were 0.01mg, 0.05mg and 0.1mg per 10μl of sterile saline. The left knee joint received an injection of saline as a placebo treatment in control mice. The knee injections were performed through the patellar tendon with the knee fixed at 90 degrees flexion [16].

Histological analysis of OA

For evaluation of cartilage degeneration and joint synovitis, samples were obtained at 2, 6, and 10 weeks after the administration of MIA (8, 12 and 16 weeks after OVX) (Supplementary Fig. 1) and were fixed in 10% neutral buffered formalin for 3 days. In addition, the samples were decalcified in 0.5M ethylenediaminetetraacetic acid (EDTA) for 5 days, and embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek, Torrance, CA, USA). Embedded samples were sectioned along the coronal plane at a thickness of 10μm. The sections of each femur and tibia were then stained with hematoxylin and eosin (H-E), and safranin-O. Slide images were captured using a Super Coolscan 5000 ED system (Nikon, Tokyo, Japan). Cartilage degeneration was evaluated using the 2010 Osteoarthritis Research Society International (OARSI) score with a scale ranging from stage 0 (normal) to 6 (vertical clefts/erosion to the calcified cartilage extending over >75% of the joint surface) for both the tibial and femoral articular cartilage on the medial side of the coronal sections for a global score of 0 to 12 for the knee joint [17]. Joint synovitis was assessed using the synovitis score with a scale ranging from 0-1 (no synovitis) to 5-9 (high-grade synovitis) for the synovial cell layer, stroma cell density and inflammatory infiltrates [18]. The synovitis grade was evaluated at the lateral synovium of the ACL and PCL on the H-E stained coronal sections. The number of osteoclasts was counted in four randomly selected fields of subchondral bone per tibia with the observer blinded to the treatment regimen received by the mice as in our previous studies [19].

Evaluation of bone micro-architecture by micro-computed tomography (μCT)

The isolated proximal tibia samples at 6 weeks after the administration of MIA (12 weeks after OVX) (Supplementary Fig. 1) were scanned on a μCT system (ScanXmate-L090, Comscantecno, Yokohama, Japan) that was operated at a lamp voltage of 75kV and a current of 100mA using X sys FP Version 1.7 and coneCTexpressIV 1.32 (Comscantecno) software. Samples were scanned at a magnification factor of 10.9 and a spatial resolution of 9.171mm/pixel. Captured images were rendered using TRI/3D BON (Ratoc System Engineering Co., Ltd., Tokyo,

Japan) software. Osteoporotic evaluation was performed on the basis of bone volume ratio (BS/BV, 1mm), bone volume fraction (BV/TV, %), trabecular number (Tb. N, 1/mm) and trabecular separation (Tb. Sp, 1μm).

Measurements of serum tartrate-resistant acid-phosphatase 5b (TRAP5b) levels

Blood samples were collected at 6 weeks after the administration of MIA (12 weeks after OVX). The samples were centrifuged at 15000 ×g for 15 min at 4˚C and the supernatants were extracted for sample preparation. The TRAP5b values were measured using a mouse TRAP5b enzyme-linked immune sorbent assay kit (Immunodiagnostic Systems, London, UK) in accordance with the manufacturer's recommendations.

Assessment of pain-like behaviors

Behavioral tests were performed prior and at 2, 4, 6, 7, 8, 9, 10, 11 and 12 weeks after OVX as in previous studies (Supplementary Fig. 1) [12,19]. In brief, to assess their mechanical withdrawal response (von Frey test), the mice were placed in plastic chambers above a wire mesh floor, which allowed full access to the hind-paw. A 1.34 g von Frey filament (Semmes-Weinstein Monofilaments, North Coast Medical Inc., San Jose, CA, USA) was used to produce mechanical tactile stimuli, which were applied to the middle area of the plantar surface of the left hind-paws. Each hind-paw was probed by 10 consecutive stimulations, while alternating between the right and left paws for each set [19]. This was repeated at least 5 times at intervals of at least 10 min and the final value was obtained from the average of the 5 measurements. Mechanical sensitivity was evaluated as the rate of withdrawal responses. Visible lifting of the stimulated hind limb was considered to be a withdrawal response [19,20]. Thermal nociceptive testing (paw-flick test) was conducted using an analgesimeter (Plantar test 7370, Ugo Basile, Italy). The mice were placed in plastic chambers (6 × 4 × 4 cm) and left unrestrained. Radiant heat was applied to the plantar surface of the left hind-paws until they were actively withdrawn by the animal. Paw withdrawal latency (PWL) was considered to be an index of the thermal nociceptive threshold, and a decrease in this measurement indicated thermal hyperalgesia. Light beam intensity was adjusted so that the basal PWL was 1-5s. The cut-off time was set at 15 s to avoid tissue damage [12]. The observer was blinded to the treatment regimen received by the mice.

Evaluation of the expression of osteoblast and osteoclast differentiation regulators

Mice were anesthetized by an intraperitoneal injection of pentobarbital and isoflurane and the right femurs were excised. The femurs were homogenized in 1ml TRIZOL reagent (Invitrogen, Carlsbad, CA) 10 times at 3,000 rpm/30 s using a bead crusher (TITEC, Tokyo, Japan). The homogenates were then centrifuged at 15,000 × g for 15 min at 4℃ and the supernatants were extracted. Total RNA was isolated from the whole femurs and reverse transcribed into cDNA by polymerase chain reaction (PCR) using an RNA PCR kit (PrimeScript^{TM} RT-PCR Kit; Takara Bio Inc., Tokyo, Japan) according to the manufacturer's protocol. The following primers were used: for Gapdh, ACCACAGTCCATGCCATCAC (forward primer) and TCCACCACCCTGTTGCTGA (reverse primer); for Runx2, GCTTGATGACTCTAAACCTA (forward primer) and AAAAAGGGCCCAGTTCTGAA (reverse primer); for Osterix, AGGCACAAAGAAGCCATAC (forward primer) and AATGAGTGAGGGAAGGGT (reverse primer); for Osteocalcin, CTCACTCTGCTGGCCCTG (forward primer) and CCGTAGATGCGTTTGTAGGC (reverse primer); and for Rankl, GGTCGGGCAATTCTGAATT (forward primer) and

GGGAATTACAAAGTGCACCAG (reverse primer) [15,21]. Reactions were carried out by PCR as follows: for Gapdh; 30 cycles at 95˚ for 40s, 55˚ for 40 s and 72˚ for 40s; for Runx2; 35 cycles at 94˚ for 30s, 60˚ for 30s, and 72˚ for 60s; for Osterix; 40cycle at 94˚ for 30s, 58˚ for 30s and 72˚ for 40s; for Osteocalcin; 35 cycles at 94˚ for 30s, 62˚ for 30s and 72˚ 30s; and for Rankl; 35 cycles at 94˚ for 30s, 65˚ for 30s and 72˚ for 60s. The values of the target gene bands were normalized to the level of Gapdh gene expression in the same sample for semi-quantitative measurement. Values in graphs are the means ± SD obtained from 4 independent experiments.

Administration of drugs

Alendronate (0.02 mg/kg of body weight diluted in physiological saline) (ALN; Merck & Co. Inc., NJ), a potent anti-resorptive agent, was administered to the mice subcutaneously [12] once a day from 6 to 8 weeks after OVX (Supplementary Fig. 1). We also examined the effect of a Cox2 inhibitor (carprofen; Sigma–Aldrich Japan, Tokyo, Japan) on pain-like behaviors in the OA model mice. Carprofen (5mg/kg of body weight diluted in physiological saline) was administered to the mice subcutaneously [14] once a day for the same period as the administration of alendronate (Supplementary Fig. 1).

Statistical analysis

All data are presented as means ± standard deviations. To determine differences between groups, measurements were repeated at least three times for each sample, and the individual mean value was used for the statistical analysis. The statistical significance between 2 groups was determined using a Student's t-test, and that among 3 or more groups was determined using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. Analyses were performed using IBM SPSS Statistics version 22.0. Differences with p values <0.05 were considered to be statistically significant.

Results

Histological analysis of OA induced by MIA in OVX mice

First, we observed cartilage degeneration in the joint after the administration of MIA at a dose of 0.01mg, 0.05mg, or 0.1mg per 10μl of sterile saline. Mild OA changes were induced at 18 weeks even by treatment with 0.01mg MIA (data not shown). Based on these results, we found that a dose as low as 0.01mg MIA induced mild OA changes at 2, 6, and 10 weeks after MIA injection, which was recognized as a model of mild OA, with progressive and significant increases in the OARSI score (data not shown).

The histological findings revealed the progression of cartilage degeneration at 2, 6 and 10 weeks after injection in the OA model mice independent of OVX (OA with osteoporosis) or not (OA). On the other hand, there were no significant histological changes in the control mice with (osteoporosis) or without OVX (control) (Fig. 1A).

The OARSI scores in the OA (n = 4) and OA with osteoporosis (n = 4) mice were significantly higher at 2, 6 and 10 weeks after MIA injection (8, 12 and 16 weeks after OVX) than in the control (n = 4) and osteoporosis (n = 4) mice, respectively. The scores increased in the OA and OA with osteoporosis mice in a time-dependent manner. There was no significant difference in the score between the OA and OA with osteoporosis mice, or between the control and osteoporosis mice (Fig. 1B).

The synovitis score was significantly increased at 2 weeks after MIA injection (8 weeks after OVX) compared to those in the control and osteoporosis mice, while there was no significant difference in the score between the OA (n = 4) and OA with osteoporosis (n = 4) mice, or between the control (n = 4) and osteoporosis (n = 4) mice (Supplementary Fig. 2). At 6 and 10 weeks (at 12 weeks and 16 weeks after OVX), there were no significant differences in synovitis score among the control, OA, osteoporosis and OA with osteoporosis mice (Supplementary Fig. 2).

Serum tartrate-resistant acid-phosphatase 5b (TRAP5b) levels

The serum level of TRAP-5b (U/L) was significantly increased in the osteoporosis (n = 4) and OA with osteoporosis (n = 4) mice in comparison with the control and OA mice (n = 4) (Fig. 1C). There was no significant difference in the serum level of TRAP5b between the control and OA mice or the osteoporosis and OA with osteoporosis mice (Fig. 1C).

Osteoporotic changes in the model mice

The BS/BV, BV/TV and Tb. N values were significantly lower and Tb. Sp value significantly higher in the osteoporosis (n = 4) and OA with osteoporosis (n = 4) mice than in the control mice (n = 4), as measured by μCT of the proximal tibial bone at 6 weeks after MIA injection (12 weeks after OVX) (Figs. 2A-E). In contrast, there were no significant differences in those values between the osteoporosis and OA with osteoporosis mice (Figs. 2A-E).

Expression of osteoblast and osteoclast differentiation regulators

We examined changes in the expression of osteoblast and osteoclast regulators at 6 weeks after MIA injection (12 weeks after OVX). The expression levels of Runx2 (Figs. 3A and B), Osterix (Figs. 3A and C), and Rankl (Figs. 3A and E) were significantly increased in the osteoporosis (n = 4) and OA with osteoporosis mice (n = 4) in comparison with those in the control mice (n = 4). In contrast, there were no significant differences in the expression levels of these regulators between the osteoporosis and OA with osteoporosis mice. The expression levels of Osteocalcin (Figs. 3A and D) in the osteoporosis and OA with osteoporosis mice tended to be increased in comparison with that in the control mice, although the difference was not statistically significant.

Changes in pain-like behaviors

The OA with osteoporosis mice (n = 5) revealed significant increases in pain-like behaviors on the von Frey test (Fig. 4A) and paw-flick test (Fig. 4B) at 1, 2, 3, 4, 5, and 6 weeks after MIA injection (7, 8, 9, 10, 11 and 12 weeks after OVX) compared with the OA mice (n = 5) (b, OA with osteoporosis versus OA; * $p<0.05$, ** $p<0.01$). The OA with osteoporosis mice (n = 5) also revealed significant increases in pain-like behaviors on the von Frey test (Fig. 4A) and paw-flick test (Fig. 4B) at 1, 2, 3, 4, 5, and 6 weeks after MIA injection (7, 8, 9, 10, 11 and 12 weeks after OVX) compared with the osteoporosis mice (n = 5) (c, OA with osteoporosis versus osteoporosis; * $p<0.05$, ** $p<0.01$). The pain-like behaviors were significantly increased from 4 weeks after OVX in comparison with the control mice (n = 4). Within 3 weeks after MIA injection, the osteoporosis mice showed more severe pain-like behaviors with OA induction than did the osteoporosis mice without OA (Figs. 4A and B). On the other hand, there was no

significant increase in pain-like behaviors in the mice without osteoporosis during the same period compared with that in the control mice (Figs. 4A and B).

Effects of bisphosphonate and Cox2 inhibitor (carprofen) on histological changes

The histological findings revealed that cartilage degeneration was observed in the OA and OA with osteoporosis mice regardless of treatment with alendronate (+ ALN) or carprofen (+ carprofen), or not (+ vehicle) in comparison with that in control mice at 6 weeks after MIA injection (12 weeks after OVX). The severity of cartilage degeneration was mild and no significant differences in of the scores were observed among those mice (Fig. 5).

Effects of bisphosphonate and Cox2 inhibitor (carprofen) on pain-like behaviors

Treatment with ALN for 2 weeks significantly improved pain-like behaviors on the von Frey test (Fig. 6A) and paw-flick test (Fig. 6B) in the OA with osteoporosis mice (c, OA with osteoporosis + ALN versus OA with osteoporosis + vehicle, * $p<0.05$, ** $p<0.01$, $n = 5$), the values for which improved to similar levels to those in the control mice ($n = 5$). In addition, these effects were maintained for more than 4 weeks, even after the discontinuation of ALN treatment (Figs. 6A and B). On the other hand, ALN treatment had no significant effect in improving pain-like behaviors in the OA mice (b, OA + ALN versus OA + vehicle, $n = 5$, respectively) (Figs. 6A and B).

We evaluated the anti-inflammatory effects on pain-like behaviors in the OA and OA with osteoporosis mice by the administration of a Cox2 inhibitor (carprofen) for 2 weeks. Carprofen significantly improved pain-like behaviors in the OA and OA with osteoporosis mice; however, these effects were only partial and were limited to the 2 weeks of the drug administration period (b, OA + vehicle versus OA + carprofen; c, OA with osteoporosis + vehicle versus OA with osteoporosis + carprofen, * $p<0.05$, ** $p<0.01$, $n = 6$, respectively) (Figs. 6C and D).

Effects of bisphosphonate and Cox2 inhibitor (carprofen) on osteoblast and osteoclast differentiation

We examined the effects of ALN and carprofen on the number of osteoclasts in the subchondral field of the proximal tibia and the expression of osteoblast and osteoclast differentiation regulators in the OA mice with or without osteoporosis at 6 weeks after MIA injection (12 weeks after OVX). The number of osteoclasts (Fig. 7A) was significantly increased in the OA with osteoporosis mice ($n = 4$, ** $p<0.01$) in comparison with that in the control and OA mice ($n = 4$). ALN significantly inhibited the increase in the number of osteoclasts in the OA with osteoporosis mice ($n = 4$, ** $p<0.01$). On the other hand, there was no significant effect of ALN on the number of osteoclasts in the OA mice (Fig. 7A). The expression levels of Runx2 (Figs. 7B and C), Osterix (Figs. 7B and D) and Rankl (Figs. 7B and F) were significantly increased in the OA with osteoporosis mice ($n = 4$, * $p<0.05$, ** $p<0.01$) in comparison with those in the control mice ($n = 4$). ALN significantly inhibited the expression levels of those regulators in the OA with osteoporosis mice ($n = 4$). The expression of Osteocalcin (Figs. 7B and E) also tended to be increased in the OA with osteoporosis mice and inhibited by ALN treatment, although these changes were not statistically significant. On the other hand, the expression levels of those regulators were not significantly changed in the OA mice ($n = 4$) in comparison with those in the control mice, and they were not affected by ALN treatment ($n = 4$). Carprofen had no significant effect on the number of osteoclasts or the expression levels of those regulators in the OA mice with and without osteoporosis (Fig. 7).

Discussion

OA is a major skeletal disease that progressively worsens over time with articular cartilage degradation, and the major source of disability for patients with OA is skeletal pain [22]. Previous studies demonstrated that the causes of pain in the pathophysiology of OA were related to increases in cytokines, chemokines and inflammatory factors in the synovium [10,11], changes in the metabolism of subchondral bone tissue [10,11], and bone marrow edema [9,10]. In addition, a recent study showed that the high metabolic activity of bone marrow lesions was associated with pain in patients with OA [23].

Our previous studies demonstrated that a high bone turnover state under osteoporotic changes involves a decrease in the threshold of pain-like behaviors and a significant relationship between bone resorption maker levels and pain threshold values in osteoporosis model mice. We also showed one of the mechanisms for the decrease in the pain threshold is the formation of an acidic microenvironment, with the increased expression of ATP and inflammatory cytokines stimulating nociceptors, such as transient receptor potential channel vanilloid subfamily member 1 (TRPV1), acid-sensing ion channels (ASICs) and P2X [12, 14, 15, 19, 21], in the bone tissue. We, then, speculated that the decreased threshold of skeletal pain due to those pathophysiological changes under osteoporotic changes could affect the induction of joint pain due to OA.

In the present study, we established mild OA model mice through the injection of low-dose (0.01mg/10μl) MIA into the joint, and showed that the pain-like behaviors in the OA mice accompanying a high bone turnover state under osteoporotic changes were significantly increased in comparison with those in mice without the osteoporotic changes. Additionally, in the early phase (within 3 weeks) after MIA injection, we did not find any significant increase in the pain-like behaviors in the mild OA model mice without the osteoporotic changes in comparison with those in the control mice. On the other hand, the OA mice with a high bone turnover state under the osteoporotic change revealed a significant increase in pain-like behaviors even within 3 weeks after MIA injection compared with the osteoporosis model mice. Interestingly, there was no significant difference in the OA grade or severity of synovitis between the mice with and without the osteoporotic changes. These results indicated that a high bone turnover state under osteoporotic changes could significantly decrease the pain threshold in mice even when pathological OA changes were mild. We, therefore, believe that the decrease in the pain threshold due to a high bone turnover state might be one of mechanisms by which the pathophysiological changes under osteoporosis affects the induction of skeletal pain in patients with OA. Furthermore, this mechanism could explain why postmenopausal female patients had more severe pain than did male patients, even when they had the same radiographic OA grade [5,6].

Previous studies indicated that pathological changes in the subchondral bone due to osteoporosis was involved in the progression of OA and induction of pain [10,11]. In this study, we used a milder OA model compared with the previous studies [16,24], in which histopathological lesions could be mainly limited to the articular cartilage. In addition, the severity of the degenerative changes in those lesions in the OA mice with osteoporotic change did not significantly differ from that in the mice without that. We, therefore, believed that the decrease in the pain threshold due to a high bone turnover state under osteoporotic changes was a significant factor in the enhancement of pain-like behaviors even in the mild OA model mice.

A number of studies reported the effects of bisphosphonate, a potent anti-bone resorption agent, on improvement in pain among OA patients through the regulation of subchondral bone metabolism, cartilage degradation, and bone

marrow lesions [25-28]; however, a certain mechanism underlying this effect remains unknown [25,26]. In this study, we did not find that bisphosphonate had any significant effect on the pain-like behaviors of the mild OA model mice without osteoporotic changes. We think that one reason for these contradictory results concerning the effect of bisphosphonate might be due to the mildness of the OA in our mouse model, with the subchondral bone lesions undergoing only mild pathological changes compared with those in the previous study, although we did not evaluate the pathohistological changes in the subchondral bone. On the other hand, in the OA model mice with osteoporotic changes, bisphosphonate markedly improved the pain-like behaviors accompanied with improvement in the high bone turnover state. Our previous studies demonstrated that bisphosphonate improved the threshold of pain-like behaviors through restoration of the high bone turnover state in osteoporosis model mice [12,14,15]. Based on these findings, we speculated that bisphosphonate might more effectively improve skeletal pain in OA patients with osteoporosis in comparison with that in patients without osteoporosis through improvement of the decreased pain threshold induced under a high bone turnover state.

Interestingly, the effect of bisphosphonate on the pain-like behaviors in the OA mice with osteoporosis was markedly decreased to a level similar to that in the control mice; however, bisphosphonate had no significant effect on the behaviors in the OA mice without osteoporosis. A recent study demonstrated that activated osteoclasts in the subchondral bone of OA joints induced sensory innervation and skeletal pain [29]. Based on the results of that and our current study, we hypothesized that the activated osteoclasts in a high bone turnover state under osteoporotic changes might induce sensory innervation more intensively than in the absence of osteoporotic changes. Additionally, our previous studies reported that continuous nociceptive stimulation due to a high bone turnover condition could induce sensitization in osteoporosis model mice [12,14]. On the other hand, in our mild OA model mice without osteoporotic changes, we speculated that the sensory innervation and continuous nociceptive stimulation might have been absent due to subchondral bone osteoclasts not being activated. In this study, we demonstrated that the number of osteoclasts was significantly increased in the OA with osteoporosis mice in comparison with that in the control and OA mice. In addition, bisphosphonate significantly inhibited the increase in the number of osteoclasts in the OA with osteoporosis mice; however, it had no significant effect on that in the OA mice. Furthermore, the pain-like behaviors in the mild OA mice without osteoporosis might be mainly related to increases in cytokine, chemokine and inflammatory factor levels in the synovium [10,11]. Thus, bisphosphonate might markedly improve the pain-like behaviors in the OA model mice with osteoporotic changes to a level similar to that in the control mice, whereas bisphosphonate did not have an effect on the model mice without osteoporotic changes. However, this study did not provide sufficient results to support our hypothesis. Thus, further studies are needed to elucidate the mechanisms by which bisphosphonate more effectively improves the pain-like behaviors due to the pathophysiological changes related to OA under osteoporotic changes than that without the condition.

Several studies indicated that a decrease in estrogen due to OVX increases skeletal pain by augmenting the excitability of the nociceptive pathway in the nervous system [30,31], and that bisphosphonate might directly suppress the release of neurotransmitters or inflammatory mediators in the nervous system [32,33]. However, most of the administered bisphosphonate is taken up by bone tissue within 24 h and remains in the bone long term [34]. In addition, our study demonstrated that the effect of ALN on improving pain-like behaviors was maintained for more than 4 weeks, even after the discontinuation of ALN treatment. We, therefore, believe that the pain-like behaviors

associated with OVX or the improvement in those behaviors by treatment with ALN were likely to reflect an increased or improved bone turnover state, although we cannot rule out the direct suppressive effects of bisphosphonate on the nervous system.

The present study had several limitations. First, the tests for pain-like behaviors such as the von Frey test and paw-flick test have not yet been established as surrogate methods for measuring skeletal pain and were not focused on the knee pain, although those tests were used for the objective and quantitative assessment of changes in animal behaviors due to skeletal pain in a number of previous studies [35,36]. Second, we did not examine whether a high bone turnover state under osteoporotic changes affects the induction of pain-like behaviors in the advanced stage of OA, which presents with subchondral bone lesions. Third, we did not evaluate the effects of anti-resorptive agents other than ALN on pain-like behaviors in our OA model. Fourth, we did not examine the effects of treatment with bisphosphonate for periods longer than 14 days. Finally, we did not evaluate changes in anti-inflammatory cytokine expression in the joint or of several differentiation markers in the nerves.

In conclusion, we demonstrated that the pain-like behaviors in the mild OA model mice with a high bone turnover state under osteoporotic changes were significantly increased in comparison with the mild OA model mice without osteoporotic changes. In addition, bisphosphonate improved the pain-like behaviors in the OA mice with osteoporotic changes accompanied by suppression of the increases in bone metabolic markers. However, bisphosphonate had no significant effects on these parameters in the mild OA model mice without osteoporotic changes. Thus, we believe that a high bone turnover state affected the induction of pain-like behaviors due to the decreased threshold of skeletal pain in the mild OA model mice.

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Author contributions

KK performed all the experiments and data analysis. KI advised and supervised the data analysis and helped to draft the manuscript. MH, KI and HH assisted with the behavioral study. AT assisted with the histological analysis of knee osteoarthritis. ME assisted with the bone morphometry and bone metabolic markers. TY participated in coordinating the study and helped to draft the manuscript. All authors read and approved the final submitted manuscript.

Conflict of interest

No authors have any conflicts of interest.

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Figure legends

Figure 1. Histological analysis of cartilage degeneration and serum levels of tartrate-resistant acid-phosphatase 5b (TRAP5b) in the model mice.

Cartilage degeneration (arrows) was found at 2, 6 and 10 weeks after MIA injection (8, 12 and 16 weeks after OVX) (A). Right panels show higher magnification views of the boxed areas in the left panels. Bars in the left panels, 500 μm; in the right panels, 100 μm.

The Osteoarthritis Research Society International (OARSI) scores in OA (grey circles) and OA with osteoporosis (black squares) mice were significantly increased at 2, 6, 10 weeks after MIA injection in comparison with those in the control (white triangles) and osteoporosis (white rhombuses) mice (B). ** $p<0.01$, OA versus control; †† $p<0.01$, OA with osteoporosis versus osteoporosis

One-way analysis of variance followed by Tukey's post hoc test.

Control, no MIA injection without OVX; OA, MIA injection without OVX; osteoporosis, no MIA injection after OVX; OA with osteoporosis, MIA injection after OVX.

The serum levels of TRAP5b was significantly increased in the osteoporosis and OA with osteoporosis mice compared with that in the control mice (C). There was no significant difference in the TRAP5b level between the osteoporosis and OA with osteoporosis mice.

* $p<0.05$, versus control mice. One-way analysis of variance followed by Tukey's post hoc test.

The sham operation was performed in all control and OA mice.

Figure 2. Osteoporotic changes in the model mice.

The μCT findings (A-E) showed osteoporotic changes in the model mice. The BS/BV (B), BV/TV (C) and Tb. N (D) values in osteoporosis mice and OA with osteoporosis mice were significantly decreased and that of the Tb. Sp (E) significantly increased in comparison with those in control mice at 6 weeks after MIA injection (12 weeks after OVX). There were no significant differences in those values between the osteoporosis and OA with osteoporosis mice (B, C, D and E). * $p<0.05$, ** $p<0.01$; versus control mice

One-way analysis of variance followed by Tukey's post hoc test.

BS/BV, bone volume ratio (1mm); BV/TV, bone volume fraction (%); Tb. N, trabecular number (1/mm) and Tb. Sp, trabecular separation (1μm).

The sham operation was performed in all control mice.

Figure 3. Expression levels of Runx2, Osterix, Osteocalcin and Rankl in the femoral bone.

The expression levels of Runx2 (A and B), Osterix (A and C), Osteocalcin (A and D) and Rankl (A and E) in the femoral bone were evaluated by semi-quantitative PCR analysis at 6 weeks after MIA injection (12 weeks after OVX). The expression levels of Runx2, Osterix and Rankl were significantly increased in the osteoporosis and OA with osteoporosis mice in comparison with the control mice, while no significant differences were observed between the osteoporosis and OA with osteoporosis mice. The expression levels are shown as the ratio of the gene of the interest to the control gene (Gapdh), and compared using those values.

* $p<0.05$, versus control mice. One-way analysis of variance followed by Tukey's post hoc test.

The sham operation was performed in all control mice.

Figure 4. Assessment of pain-like behaviors.

The pain-like behaviors on the von Frey test (A) and paw-flick test (B) after OVX and MIA injection

The OA mice (grey circles) showed significant increases in pain-like behaviors from 4 weeks after MIA injection (10 weeks after OVX) compared with the control mice (white triangles) (a; * $p<0.05$, ** $p<0.01$, versus control mice). The osteoporosis mice (white rhombuses, black squares) showed significant increases in pain-like behaviors from 4 weeks after OVX (a; * $p<0.05$, ** $p<0.01$, versus control mice). The OA with osteoporosis (black squares) mice showed significant increases in pain-like behaviors from 1 week after MIA injection (7 weeks after OVX) in comparison with OA mice (grey circles) (b; ** $p < 0.01$, versus OA mice). The OA with osteoporosis mice (black squares) also showed significant increases from 1 week after MIA injection (7 weeks after OVX) in comparison with osteoporosis mice (white rhombuses) (c; * $p<0.05$, ** $p<0.01$, versus osteoporosis mice). One-way analysis of variance followed by Tukey's post hoc test.

The sham operation was performed in all control and OA mice.

Figure 5. Effects of alendronate and Cox2 inhibitor (carprofen) on histological changes.

Histological features (arrows, cartilage degeneration) at lower (left) and higher (right) magnifications at 6 weeks after MIA injection (12 weeks after OVX). Bars in the left panels, 500 μm; in the right panels, 100 μm (A)

The Osteoarthritis Research Society International (OARSI) scores in the OA and OA with osteoporosis mice were significantly increased independent of the treatment with alendronate or carprofen in comparison with the control mice. There was no significant difference in the OARSI score between the two groups of OA mice (B).

** $p<0.01$, OA versus control mice. One-way analysis of variance followed by Tukey's post hoc test.

The sham operation was performed in all control and OA mice.

Figure 6. Effects of alendronate (ALN) and Cox2 inhibitor (carprofen) on pain-like behaviors.

Treatment with ALN (white squares) significantly improved pain-like behaviors on the von Frey test (A) and paw-flick test (B) in the OA with osteoporosis mice (black squares), the values for which improved to levels similar to those in the control mice (white triangles). These effects were maintained for more than 4 weeks after the discontinuation of treatment (A, B). On the other hand, ALN (white circles) did not improve pain-like behaviors in the OA mice (grey circles) (A, B).

Carprofen (white circles, white squares) significantly improved the pain-like behaviors in the OA and OA with osteoporosis mice (grey circles, black squares) (C, D). These changes were only partial and limited to the 2 weeks of drug administration (white circles, white squares). One-way analysis of variance followed by Tukey's post hoc test.

* $p<0.05$, ** $p<0.01$; a, versus control mice; b, versus OA mice; c, versus OA with osteoporosis mice + ALN and/or + carprofen

The sham operation was performed in all control and OA mice.

Figure 7. Effects of alendronate (ALN) and Cox2 inhibitor (carprofen) on the number of osteoclasts, and the expression levels of Runx2, Osterix, Osteocalcin and Rankl in the femoral bone in OA and OA with osteoporosis mice.

The number of osteoclasts in the subchondral bone of the proximal tibia (A) and the expression levels of Runx2 (B, C), Osterix (B, D) and Rankl (B, F) in the femoral bone were significantly increased in the OA with osteoporosis mice (OA with osteoporosis + vehicle). Alendronate (OA with osteoporosis + ALN) significantly inhibited the number of that (A) and the expression levels of these regulators (C, D, F) in the mice (OA with osteoporosis + vehicle). In contrast, alendronate (OA + ALN) had no significant effect on the number of osteoclasts (A) and the expression levels of these regulators (C – F) in the OA mice (OA + vehicle). Carprofen had no significant effect on the number of osteoclasts or the expression in the OA mice with and without osteoporosis.

The expression levels are shown as the ratio of the gene of the interest to the control gene (Gapdh), and compared using those values. One-way analysis of variance followed by Tukey's post hoc test. * $p<0.05$, ** $p<0.01$

The sham operation was performed in all control and OA mice.

Supplementary Figure 1. Study design.

The 8-week-old mice were bilaterally ovariectomized (OVX), and monosodium iodoacetate (MIA) was administrated to the left knee joint at 6 weeks after OVX. Pain-like behaviors were assessed at 0, 2, 4, 6, 7, 8, 9, 10, 11 and 12 weeks after OVX. Histological analysis was performed at 2, 6 and 10 weeks after MIA injection (8, 12 and 16 weeks after OVX). Bone micro-architectural changes (as assessed by μCT), serum levels of TRAP5b, and the expression of osteoblast and osteoclast differentiation regulators were evaluated at 6 weeks after MIA injection (12 weeks after OVX). Alendronate (ALN, 0.02 mg/kg of body weight) and Carprofen (5mg/kg of body weight) were administered to the mice subcutaneously once a day from 6 to 8 weeks after OVX.

Supplementary Figure 2. Histological evaluation of synovitis in the model mice.

Synovial tissue around the anterior cruciate ligament (ACL) and posterior cruciate ligament (PCL) (boxed area) was evaluated for lining cell layer, synovial stroma and inflammatory infiltrates (arrows) (A). The synovitis scores in the OA (grey circles) and OA with osteoporosis (black squares) mice were significantly higher at 2 weeks after MIA injection (8 weeks after OVX) than those in the control (white triangles) and osteoporosis (white rhombuses) mice. At 6 and 10 weeks after MIA injection (12 weeks and 16 weeks after OVX), there were no significant differences in the scores among the control, OA, osteoporosis and OA with osteoporosis mice (B). * $p<0.05$, OA versus control; † $p<0.05$, OA with osteoporosis versus osteoporosis by one-way analysis of variance followed by Tukey's post hoc test.

The sham operation was performed in all control and OA mice.

Right panels show higher magnification views of the boxed areas in the left panels

Bars in the left panels, 500 μm; in the right panels, 100 μm.

Fig. 1

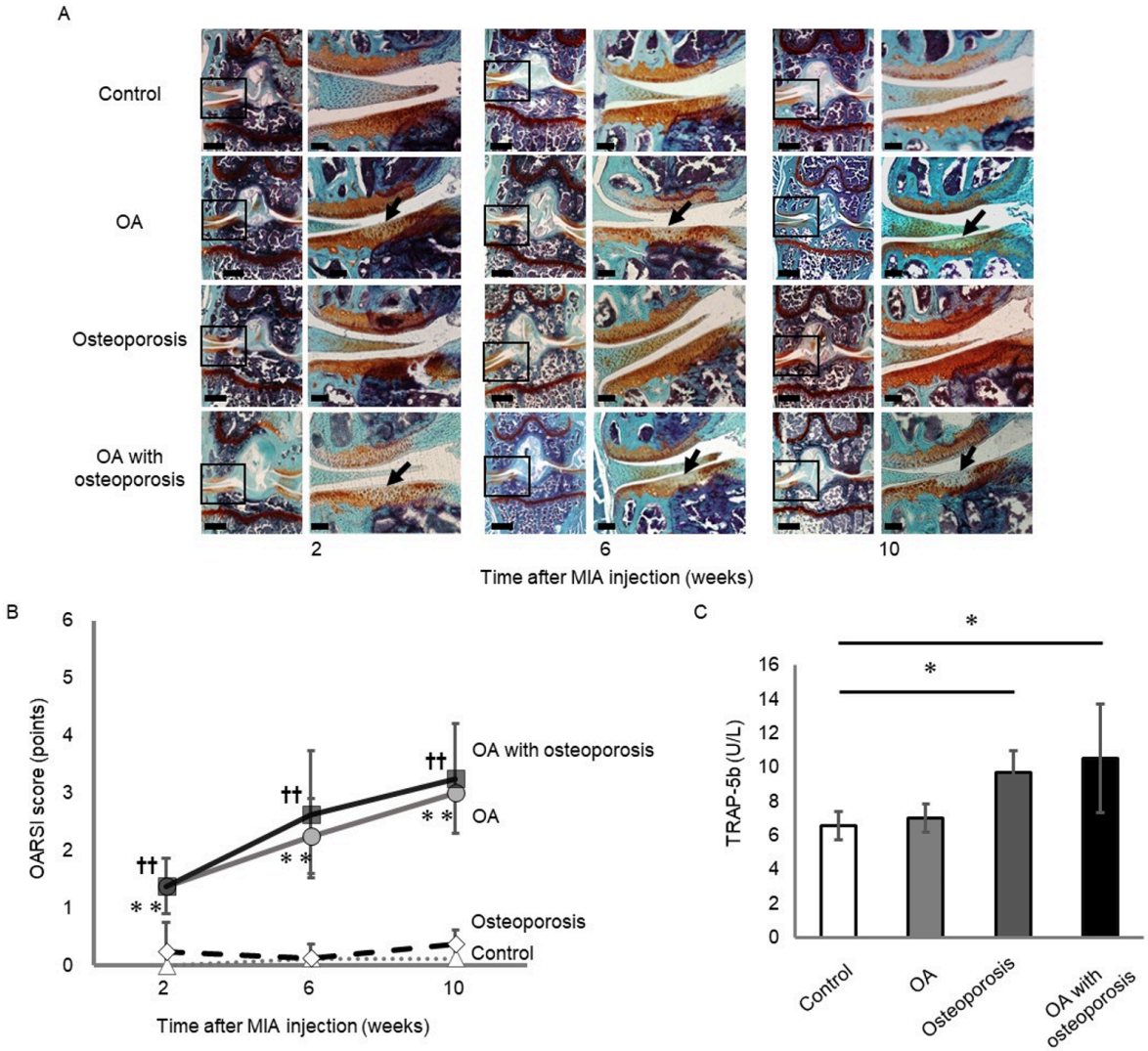

A
Control
OA
Osteoporosis
OA with osteoporosis
2
6
10
Time after MIA injection (weeks)
B
OARSI score (points)
OA with osteoporosis
OA
Osteoporosis
Control
Time after MIA injection (weeks)
C
TRAP-5b (U/L)
Control
OA
Osteoporosis
OA with osteoporosis

Fig. 2

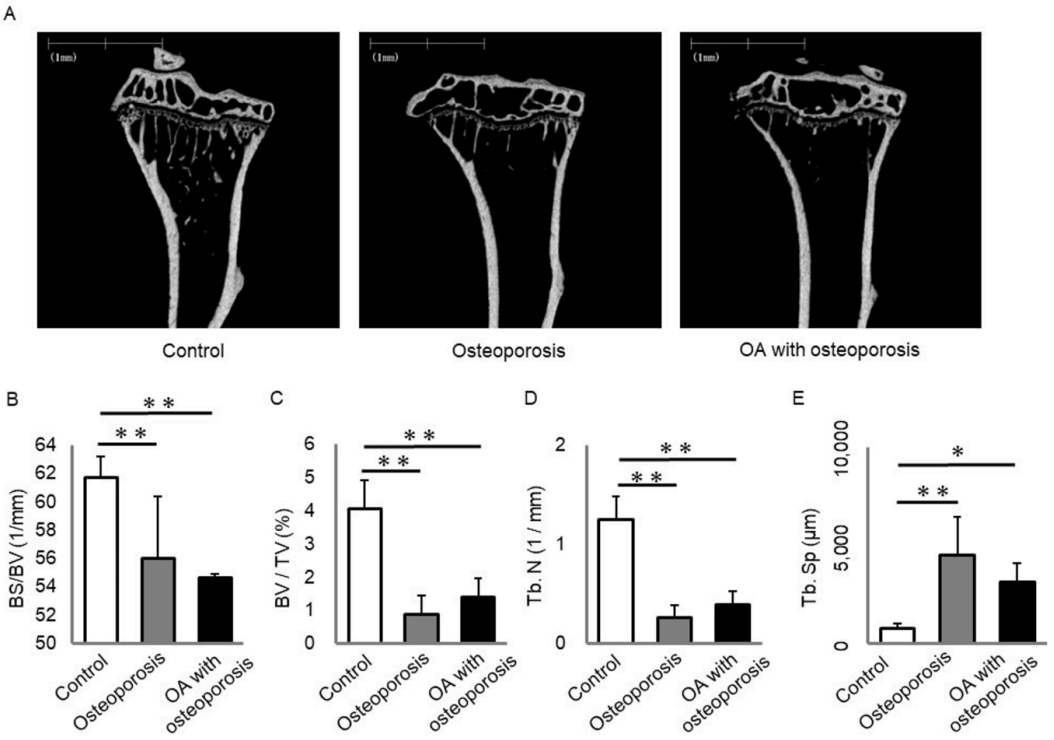

A
Control
Osteoporosis
OA with osteoporosis
B
BS/BV (1/mm)
C
BV / TV (%)
D
Tb. N (1 / mm)
E
Tb. Sp (μm)
Control
Osteoporosis
OA with osteoporosis

Fig. 3

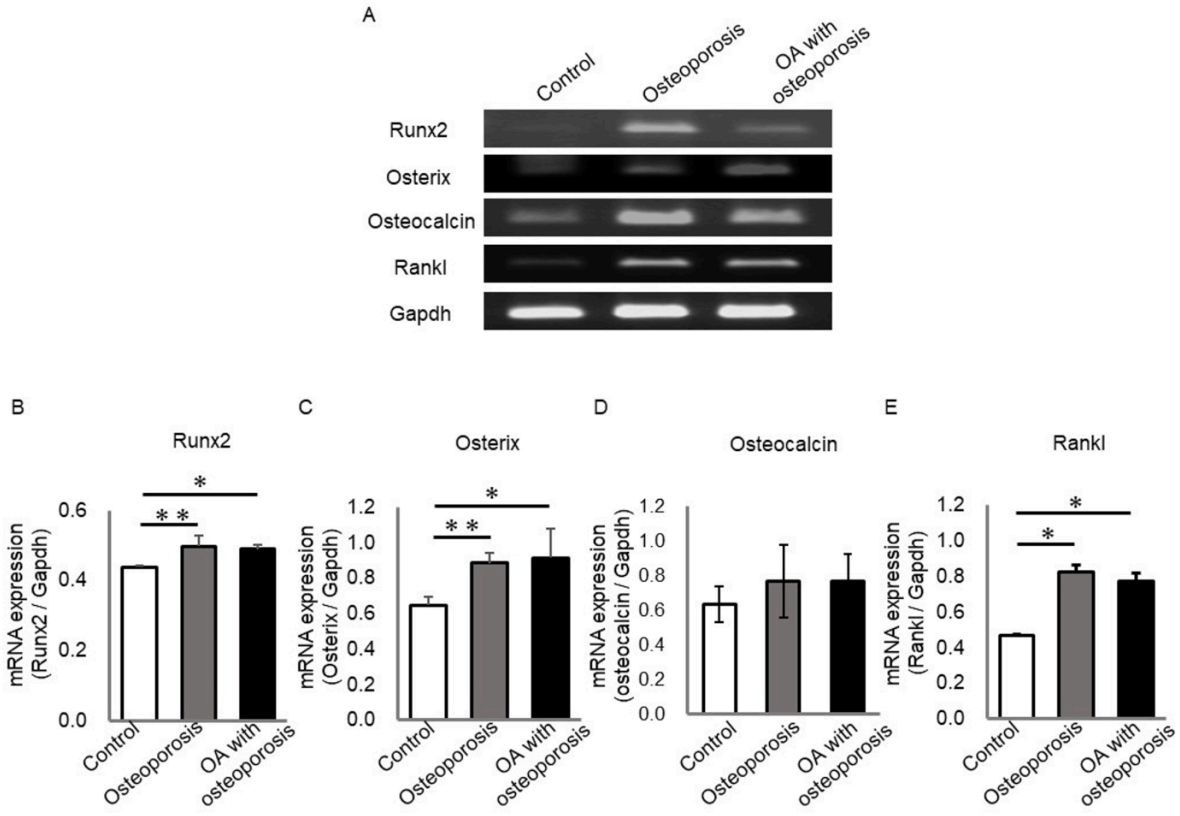

A
Control
Osteoporosis
OA with osteoporosis
Runx2
Osterix
Osteocalcin
Rankl
Gapdh
B
Runx2
mRNA expression (Runx2 / Gapdh)
C
Osterix
mRNA expression (Osterix / Gapdh)
D
Osteocalcin
mRNA expression (osteocalcin / Gapdh)
E
Rankl
mRNA expression (Rankl / Gapdh)
Control
Osteoporosis
OA with osteoporosis

Fig. 4

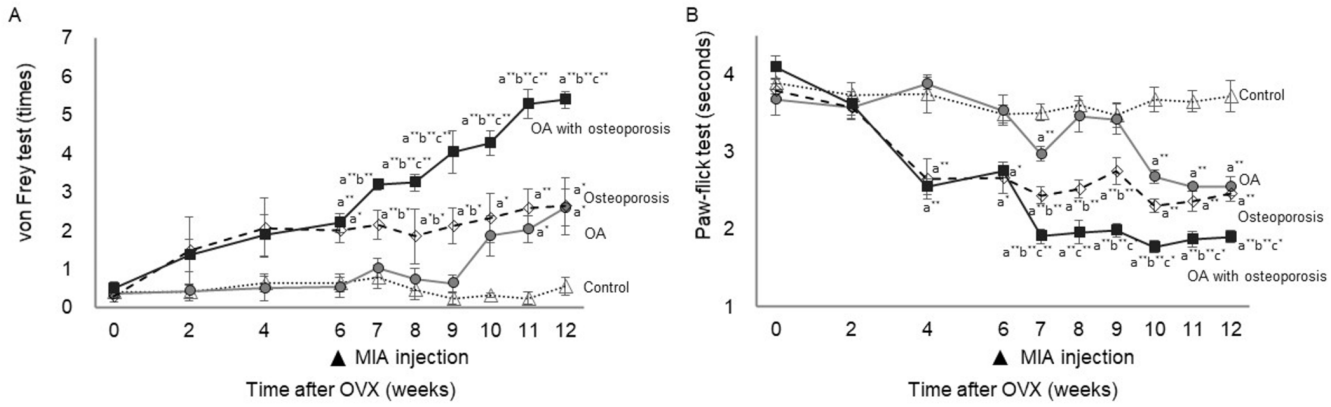

A
von Frey test (times)
OA with osteoporosis
Osteoporosis
OA
Control
▲ MIA injection
Time after OVX (weeks)
B
Paw-flick test (seconds)
Control
OA
Osteoporosis
OA with osteoporosis
▲ MIA injection
Time after OVX (weeks)

Fig. 5

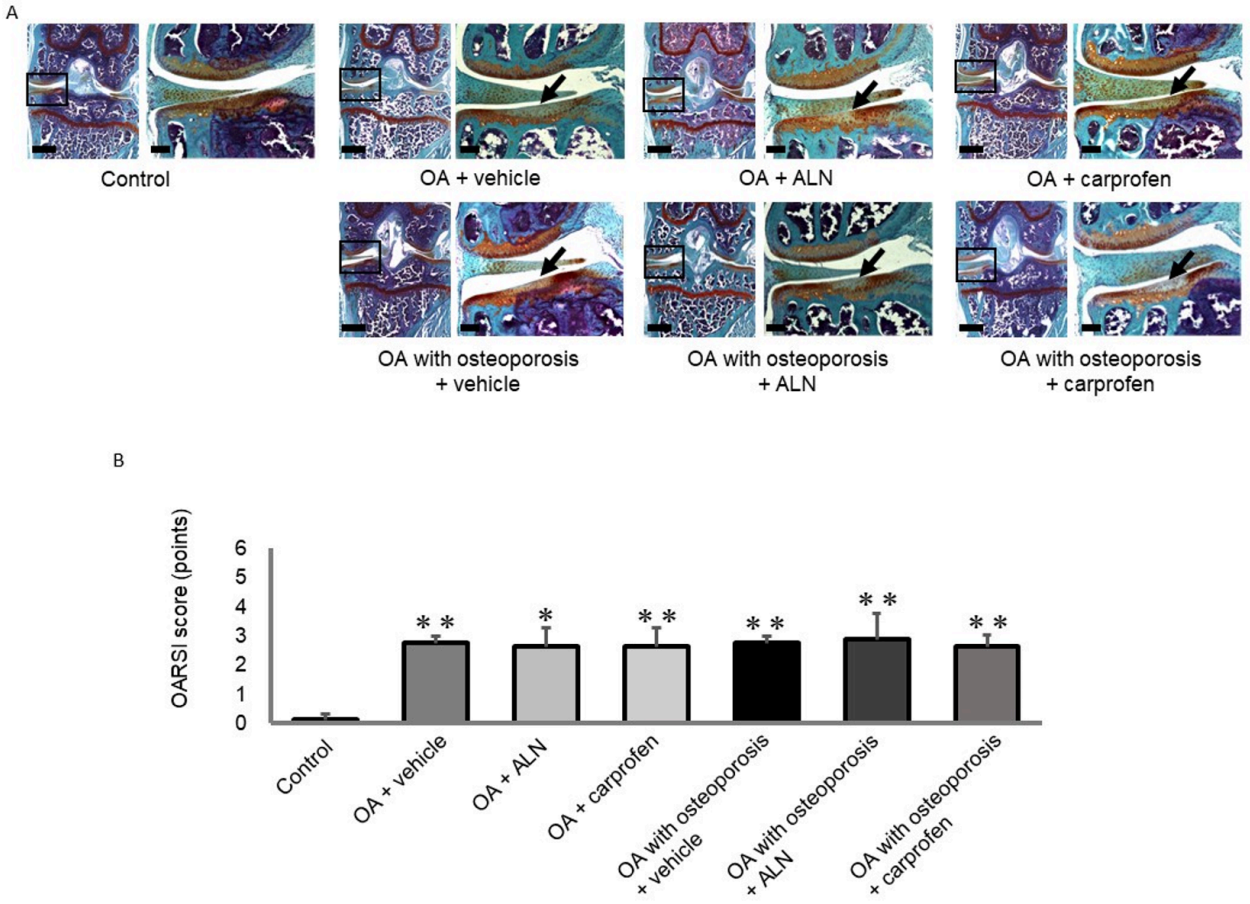

A
Control
OA + vehicle
OA + ALN
OA + carprofen
OA with osteoporosis + vehicle
OA with osteoporosis + ALN
OA with osteoporosis + carprofen
B
OARSI score (points)
0
1
2
3
4
5
6
**
*
**
**
**
**
Control
OA + vehicle
OA + ALN
OA + carprofen
OA with osteoporosis + vehicle
OA with osteoporosis + ALN
OA with osteoporosis + carprofen

Fig. 6

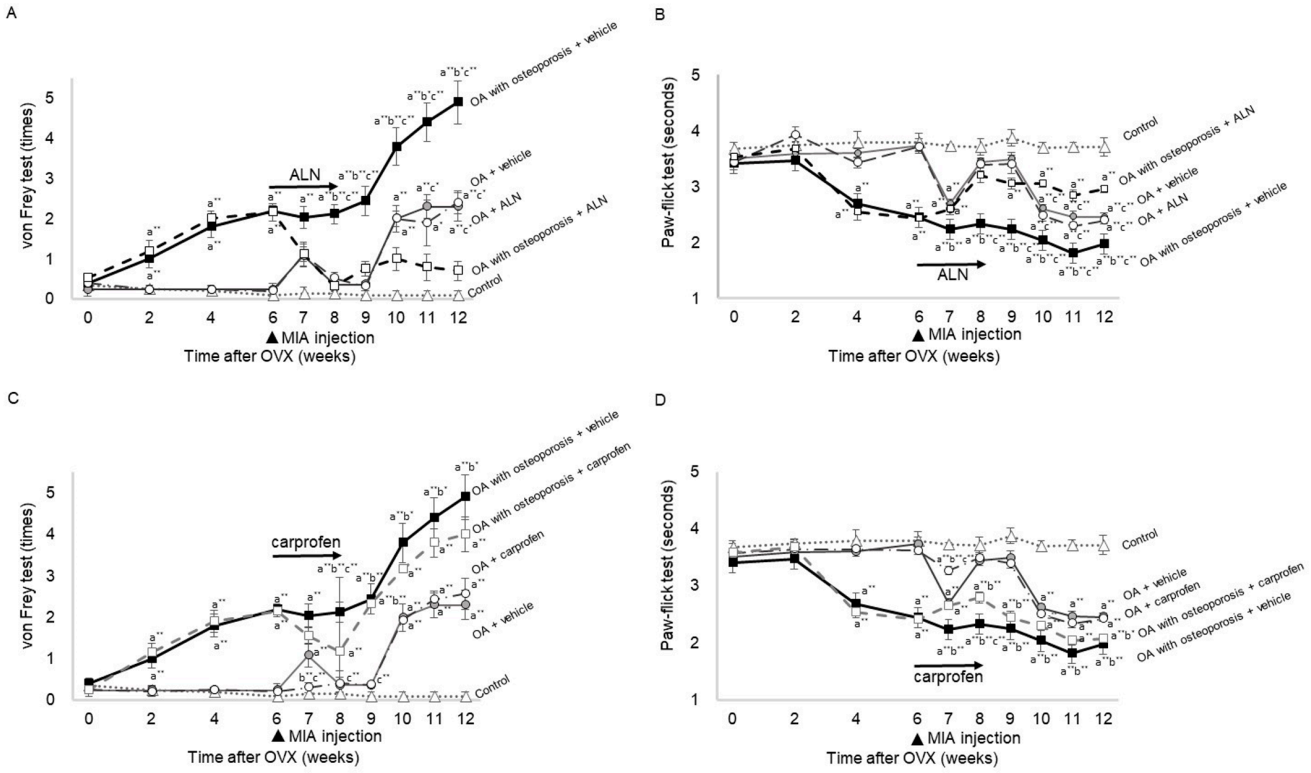
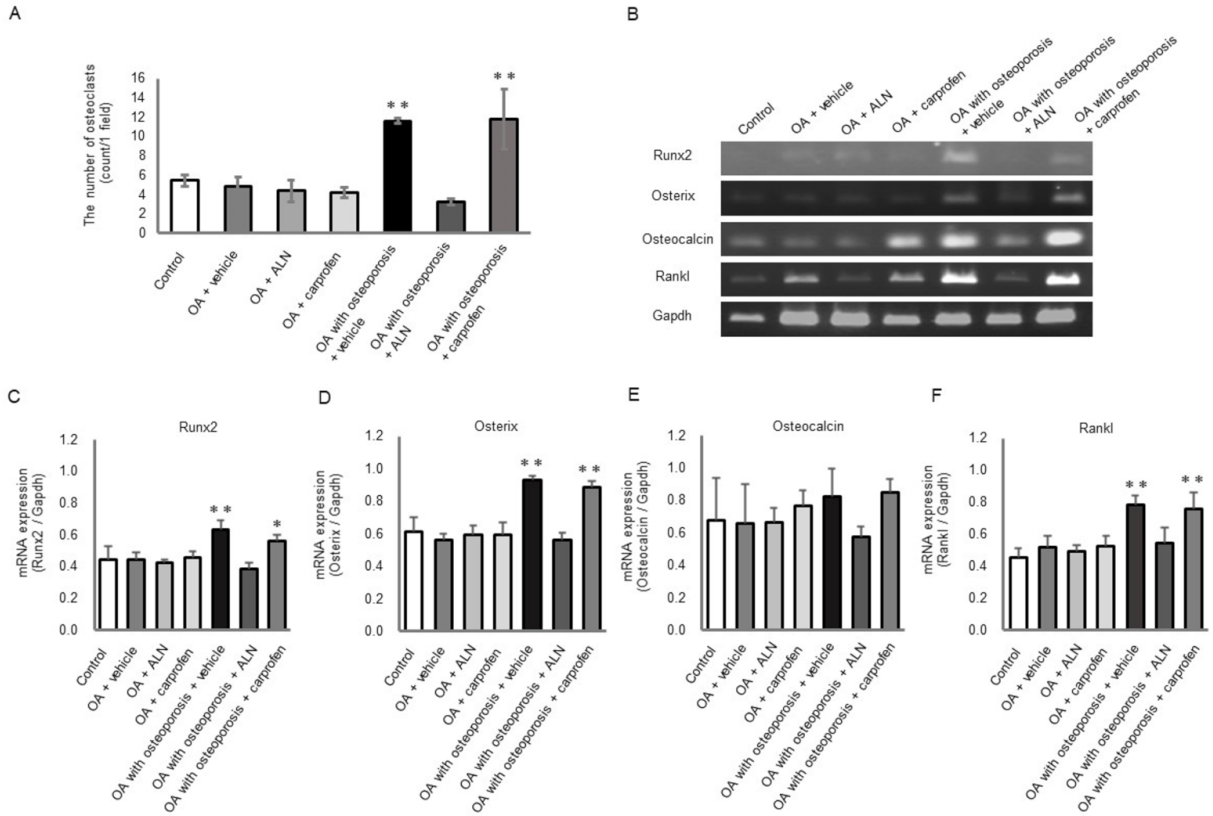
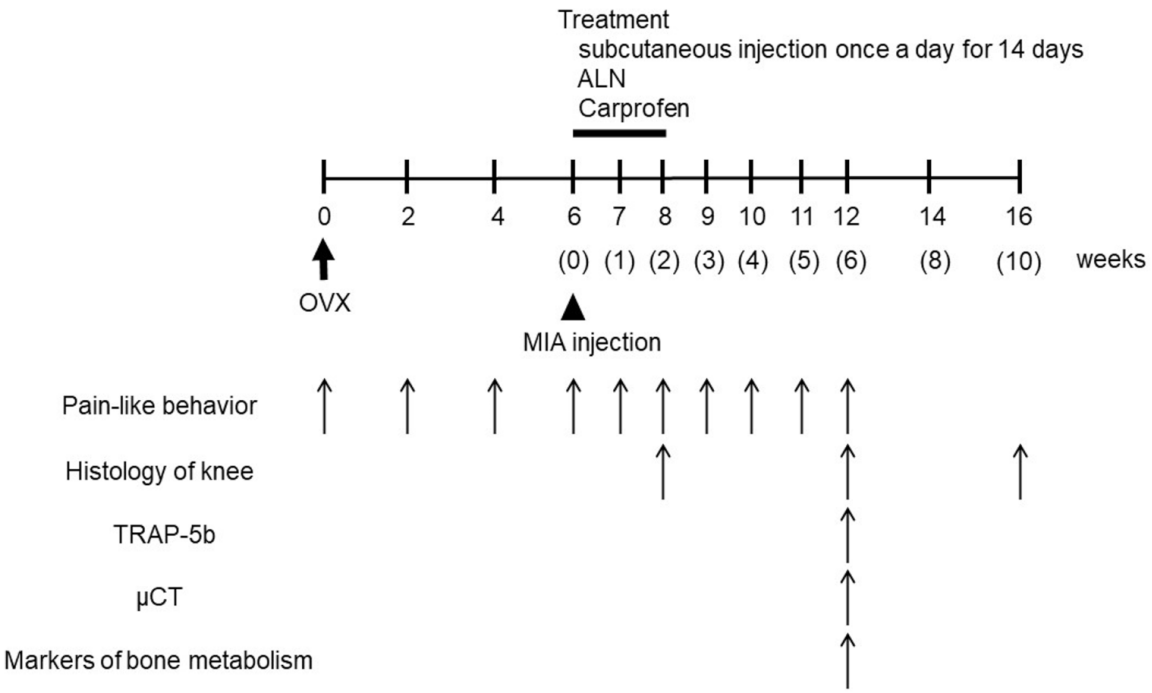

A
von Frey test (times)
ALN
OA with osteoporosis + vehicle
OA + vehicle
OA + ALN
OA with osteoporosis + ALN
Control
▲MIA injection
Time after OVX (weeks)
B
Paw-flick test (seconds)
Control
OA with osteoporosis + ALN
OA + vehicle
OA + ALN
OA with osteoporosis + vehicle
ALN
▲MIA injection
Time after OVX (weeks)
C
von Frey test (times)
carprofen
OA with osteoporosis + vehicle
OA with osteoporosis + carprofen
OA + carprofen
OA + vehicle
Control
▲MIA injection
Time after OVX (weeks)
D
Paw-flick test (seconds)
Control
OA + vehicle
OA + carprofen
OA with osteoporosis + carprofen
OA with osteoporosis + vehicle
carprofen
▲MIA injection
Time after OVX (weeks)

Fig. 7

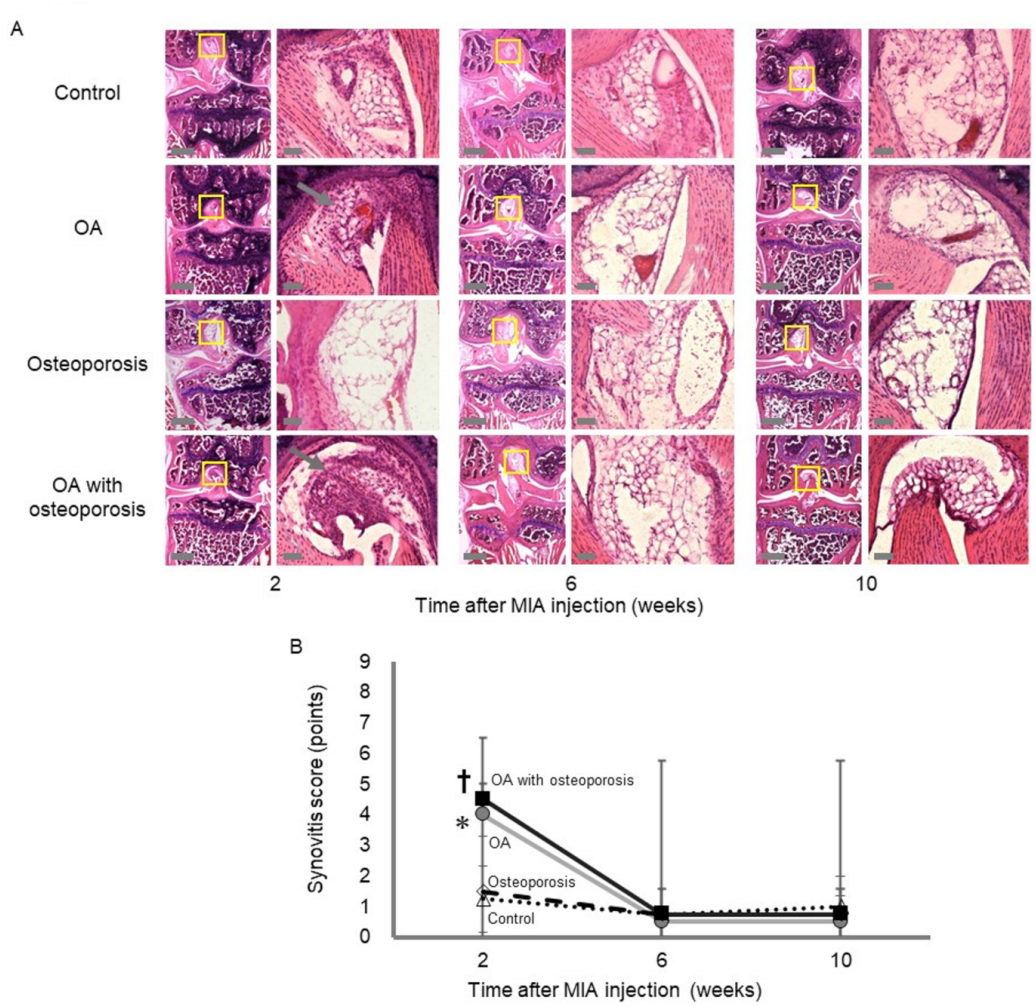

A
The number of osteoclasts (count/1 field)
Control
OA + vehicle
OA + ALN
OA + carprofen
OA with osteoporosis + vehicle
OA with osteoporosis + ALN
OA with osteoporosis + carprofen
B
Runx2
Osterix
Osteocalcin
Rankl
Gapdh
C
Runx2
mRNA expression (Runx2 / Gapdh)
D
Osterix
mRNA expression (Osterix / Gapdh)
E
Osteocalcin
mRNA expression (Osteocalcin / Gapdh)
F
Rankl
mRNA expression (Rankl / Gapdh)

Supplementary Fig. 1



High bone turnover state under osteoporotic changes induces pain-like behaviors in mild osteoarthritis model mice. *Journal of Bone and Mineral Metabolism*. Kenta Kiyomoto, Kousuke Iba, Megumi Hanaka, Koji Ibe, Hikaru Hayakawa, Atsushi Teramoto, Makoto Emori, Toshihiko Yamashita. Department of Orthopaedic Surgery, Sapporo Medical University School of Medicine. iba@sapmed.ac.jp

Supplementary Fig. 2



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