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ORIGINAL ARTICLE

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Differences in osteoclast formation between proximal and distal tibial osteoporosis in rats with adjuvant arthritis: inhibitory effects of bisphosphonates on osteoclasts

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Abstract Patients with rheumatoid arthritis commonly suffer both systemic and periarticular osteoporosis. Bisphosphonates (BPs) are inhibitors of bone resorption, and several derivatives have been developed for treatment of enhanced bone resorption. We aimed to characterize osteoclast formation in two different sites, the proximal tibial and distal tibial areas, in rats with adjuvant arthritis, and to investigate the impact of amino or non-amino types of bisphosphonate. Adjuvant arthritis was initiated in rats while administering daily injections of either etidronate, a non-amino BP, or alendronate, an amino BP, for 3 weeks. On the day following the last injection, bone mineral density (BMD) was measured in the proximal tibia to assess systemic osteoporosis and in the distal tibia for periarticular osteoporosis using dual-energy X-ray absorptiometry. Subsequently, bone marrow cells from either end of the tibia were collected and incubated for 7 days before staining and counting tartrate-resistant acid phosphatase positive cells. In the rats with adjuvant arthritis, BMD of either end of the tibia was lower than in normal rats. Although etidronate prevented bone mineral loss at both ends, distal loss was significantly less than proximal. In contrast, alendronate significantly inhibited mineral loss primarily in the proximal area. Large osteoclasts, defined as having five or more nuclei, formed preferentially in the proximal tibia, while small osteoclasts with fewer than four nuclei were found mainly distally. The suppressive effect of alendronate was greater on the large osteoclasts, while etidronate had a greater effect on the small osteoclasts. These results show that the size and multinuclearity of osteoclasts and the number of osteoclasts formed are different in the distal and proximal areas of the tibia, and that alendronate and etidronate may suppress different types of osteoclasts as discriminated by the number of nuclei.

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Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disorder characterized by joint swelling, synovial inflammation, and joint destruction. In many patients the disease is progressive, ultimately resulting in joint destruction, and so leading to significant disability. Adjuvant arthritis in rat (AA rat) shows several clinical and histological similarities to human RA. Its maximal clinical activity, 2–3 days post induction, is followed by chronic arthritis characterized by synovial hyperplasia, inflammatory infiltration, and cartilage and bone destruction.[1]

Bisphosphonates (BPs) are synthetic analogues of pyrophosphate that contain non-hydrolysable P–C–P bonds, have a high affinity for bone minerals, and inhibit bone resorption by osteoclasts.[2] These compounds have become the drugs of choice for treatment of skeletal disorders involving excessive osteoclast mediated bone resorption, such as Paget's disease, tumor-induced osteolysis and hypercalcemia, postmenopausal osteoporosis, and periarticular and systemic osteoporosis in RA.[3–7] It has been reported that some BPs inhibit bone destruction in AA rats.[8–10] Among the BPs available clinically for RA patients, etidronate is known to suppress bone resorption and destruction, and is also known to have anti-inflammatory and suppressive effects on joint destruction.[11]

Osteoporosis associated with RA may be divided into two types, systemic and periarticular. Although osteoclasts are involved in both, generalized or systemic osteoporosis results in widespread bone fragility as seen in steroidal, postmenopausal, or disuse osteoporosis and may result in fracture; In contrast, periarticular osteoporosis is not only associated with bone erosion and destruction but with inflammatory changes as well. Here, we report that these two types of osteoporosis may be morphologically distinct, and investigate whether the bisphosphonates of amino or non-

amino types may also have distinct actions on these different osteoclasts.

Materials and methods

Animals

Male Lewis rats aged 5 weeks were obtained from Charles River Japan (Kanagawa, Japan). The animals were maintained four to a cage in an environment free of stress with unrestricted access to rat chow (ED-2; Japan Crea, Japan) as well as water. The cages were placed in an air-conditioned room with a 12:12-h light/dark cycle with room temperature maintained at 23° ± 2°C and humidity at 55% ± 10%. The animals were held in quarantine for 1 week before use in the experiments.

We induced adjuvant arthritis in 6-week-old rats by subcutaneous injection into the footpad of the right hind limb of 0.2 ml of a *Mycobacterium butyricum* suspension. This was prepared by suspending heat-killed *M. butyricum* (Difco Laboratories, Detroit, MI, USA) in liquid paraffin at a concentration of 0.5%.

Testing agents

Etidronate or alendronate was dissolved in saline at appropriate concentrations. We started administration of the compounds on the day of adjuvant injection, given subcutaneously 5 days a week for 3 consecutive weeks. The animals were divided into six groups as follows: normal, adjuvant control, etidronate 5 mg/kg per day, etidronate 10 mg/kg per day, alendronate 0.025 mg/kg per day, and alendronate 0.05 mg/kg per day. A total of 60 rats were used, 10 rats per group. Etidronate 10 mg/kg per day and alendronate 0.05 mg/kg per day are both the usual clinical dosages.

Bone mineral density measurement

On the day following the last injection, tibia on the side opposite to that receiving the adjuvant injection were removed from animals killed by an overdose of ether. We measured bone mineral density (BMD) at the proximal and distal ends of the tibia (within 5 mm from either epiphysis) using dual-energy X-ray absorptiometry (DEXA) (DSC-600; Aloka, Tokyo, Japan) (Fig. 1).

Cell preparation

Cells were prepared from proximal and distal tibial areas of AA rats treated with vehicle alone. On the day after the last administration, tibial tissues were minced with scissors after the removal of skin. The minced tissue was digested for 7 h by collagenase and dispase in α-minimum essential medium (α-MEM) containing 10% fetal bovine serum (10% FCS-α-MEM; Gibco, MD, USA) at 37°C. After treatment, dispersed cells were filtered through mesh and collected. They were washed thrice and then the pellet was suspended in 5% FCS-α-MEM, and live nucleated cells were counted with a combination of Turk's stain and trypan blue solution. Cells were adjusted to 2×10^5 live cells/ml for use in the following experiments.

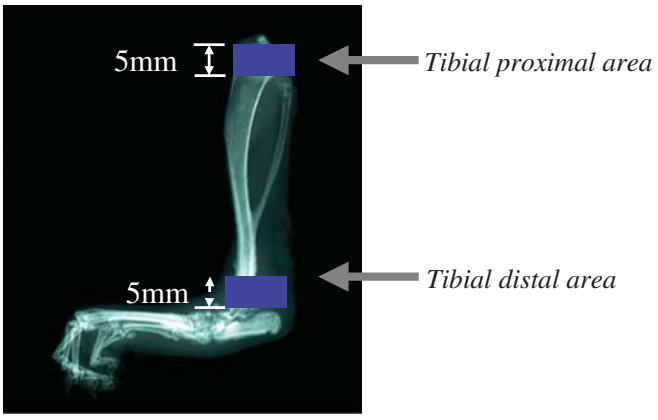

Fig. 1. Dual-energy X-ray absorptiometry measurements of bone mineral density of tibial bones on the side opposite the adjuvant injection (DSC-600; Aloka)

Cell culture[12]

Cells were introduced into 96-well plates at 0.2 ml/well and incubated with or without bisphosphonates (etidronate, 10^{-5} M and alendronate, 10^{-7} M) for 7 days at 37°C in 5% CO_2. Thereafter, cell-free supernatant was removed by aspiration and the cells were fixed with paraformaldehyde. To identify tartrate-resistant acid phosphatase (TRAP)-positive cells, we added a staining solution (50 mM sodium citrate, 100 mM sodium tartrate, 55 mM *p*-nitrophenol) to the fixed cells after washing. Cells from arthritic lesions spontaneously formed bone-resorbing osteoclast-like cells in vitro without the addition of the exogenous cytokines, macrophage colony-stimulating factor, tumor necrosis factor (TNF)-α, fibroblast growth factor-2, or hormones. TRAP-positive osteoclast-like cells, defined as dark red cells, were counted microscopically. We defined two types of osteoclasts: those with five or more nuclei and those with fewer than five. To confirm the bone-resorbing ability of osteoclast-like cells formed in this manner, we also performed a pit formation assay.

Pit formation assay

Preparation of dentine slices and the pit formation assay were modified from the method described by Takada et al.[13] Cells of arthritic animals were cultured on dentine slices (6 mm diameter) in each well of 96-well microplates for 7 days as described above. After cultivation, cells were wiped off and the pits stained with acid hematoxylin (Sigma Diagnostics, St. Louis, MO, USA). Pits were identified as dark violet spots on the dentine.

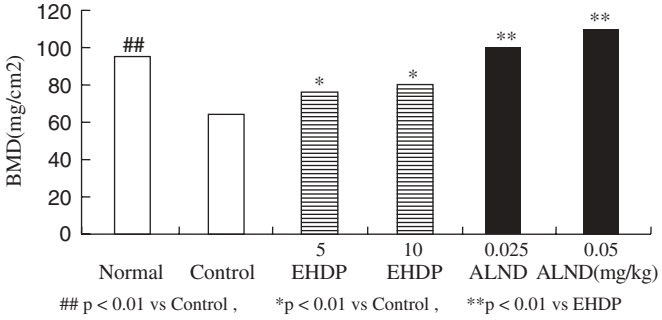

Fig. 2. Bone mineral density of the tibial proximal area. In the adjuvant arthritis rat model, bone mineral density (*BMD*) of the tibial proximal area is significantly decreased in positive controls compared to the normal group. Both the etidronate (*EHDP*) and alendronate (*ALND*) groups have significantly higher BMD than the positive control group ($P < 0.01$). In particular, the alendronate group has a significantly higher BMD than the etidronate group ($P < 0.01$)

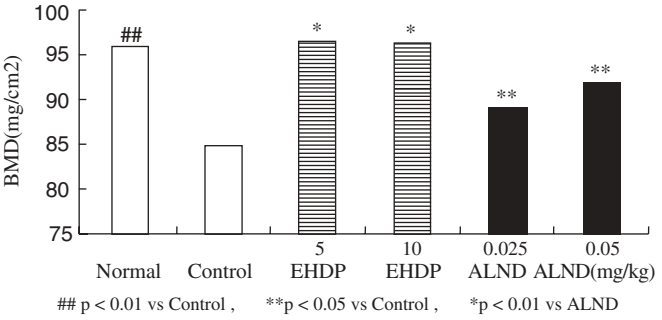

Fig. 3. Bone mineral density (*BMD*) of the tibial distal area. Bone mineral density of the tibial distal area in the control group is significantly reduced. Both the etidronate (*EHDP*) ($P < 0.01$) and alendronate (*ALND*) groups ($P < 0.05$) have higher BMD than the control group. The etidronate group shows a significantly higher BMD than the alendronate group ($P < 0.01$)

Statistical analysis

We undertook statistical analysis of BMD in the proximal and distal tibial areas using two-way analysis of variance. If the analysis results were significant at a level of 5%, we tested intergroup differences using Tukey's post hoc test for multiple comparisons. When examining tibial regions for differences in osteoclasts, we exclusively used data from the adjuvant control group. For each tibial region, we took the difference between the number of large and small osteoclasts and then tested different regions with the *t*-test.

Results

Bone mineral density at proximal and distal sites of the tibia

Significant differences in BMD of the proximal tibial area on the side opposite to that of the adjuvant injection were found among the six groups ($P < 0.01$). The normal negative controls had a BMD of $94.9 \pm 2.8\,mg/cm^2$, while the adjuvant positive control group showed a markedly lower BMD of $64.0 \pm 3.7\,mg/cm^2$ ($P < 0.01$). The alendronate groups showed a significantly higher BMD at the proximal tibial area than did the etidronate groups ($P < 0.01$) (Fig. 2): the etidronate 5 mg/kg per day group had a BMD of $76.4 \pm 4.6\,mg/cm^2$; the 10 mg/kg per day group $80.2 \pm 4.4\,mg/cm^2$, the alendronate 0.025 mg/kg per day group $99.9 \pm 3.0\,mg/cm^2$, and the 0.05 mg/kg per day group $109.7 \pm 6.0\,mg/cm^2$.

The normal group had a BMD of $95.5 \pm 2.2\,mg/cm^2$ in the distal tibial area, while the adjuvant control group, at $84.6 \pm 4.0\,mg/cm^2$, was significantly lower ($P < 0.01$). The results of the etidronate and alendronate groups showed a BMD of $96.2 \pm 1.6\,mg/cm^2$ in the etidronate 5 mg/kg per day group, $96.0 \pm 1.7\,mg/cm^2$ in the etidronate 10 mg/kg per day group, $88.9 \pm 2.0\,mg/cm^2$ in the alendronate 0.025 mg/kg per day group, and $91.4 \pm 1.5\,mg/cm^2$ in the alendronate 0.05 mg/kg per day group. The etidronate groups had significantly higher distal tibial area BMD than did the alendronate groups ($P < 0.01$) (Fig. 3).

Histological findings

The histochemistry of the bone tissues from the proximal and distal areas by hematoxylin–eosin (H&E) and TRAP staining. In normal rats, osteoclast-like cells could not be found, either in the proximal or distal tibial area, including the ankle joint. However, in AA rats small osteoclasts were identified, mainly in the distal tibial area, and large osteoclasts in the proximal tibial area (Fig. 4). H&E and TRAP staining revealed that it was mainly the small osteoclasts that were reduced in the distal tibial area in treated rats, whereas alendronate targeted mainly the large osteoclasts in the proximal tibial area (Fig. 5).

Formation of osteoclasts from the cells of proximal and distal sites of the tibia

Here, we define an osteoclast with five or more nuclei as "large," and one with fewer than four as "small" in vitro by TRAP staining (Fig. 6). The number of large osteoclasts was significantly greater than the number of small ones at the tibial proximal area in the adjuvant control group (39.3 cells/well large vs 12.3 cells/well small, $P < 0.01$) (Fig. 7a). The number of large osteoclasts at the tibial proximal area was significantly lower in the alendronate than in the etidronate group, as shown in Fig. 7b (11.0 cells/well large osteoclasts with alendronate vs 22.0 cells/well large with etidronate, $P < 0.01$), In contrast, at the tibial distal area of the adjuvant control group, the number of small osteoclasts was greater than the number of large ones (26.7 cells/well large vs 70.7 cells/well small, $P < 0.01$) (Fig. 7c). It was clear that etidronate reduced the number of small osteoclasts at the tibial distal area more than did the alendronate group, as shown in Fig. 7d (19.0 cells/well small osteoclasts with etidronate vs 54.3 cells/well small ones with alendronate, $P < 0.01$).

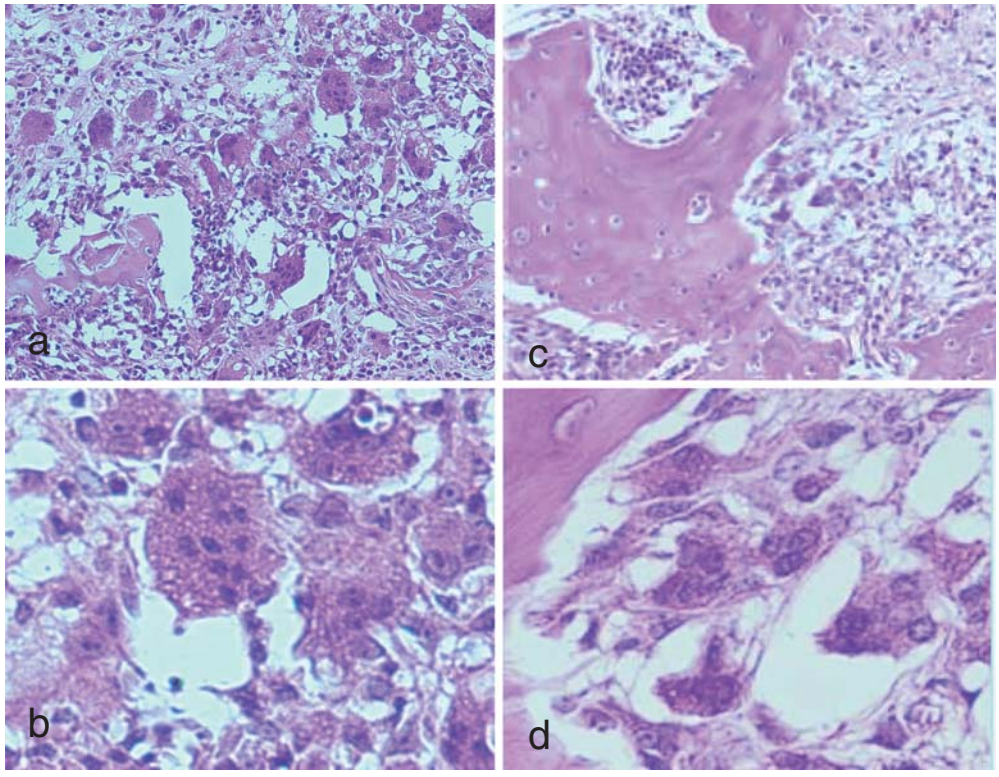

Fig. 4a–d. Histological findings at the proximal and distal tibial area in adjuvant arthritis (AA) rats. The proximal tibial area is rich in large osteoclasts (**a** and **b**), whereas the small osteoclasts are mostly found at the distal tibial area (**c** and **d**). (H&E and TRAP staining; **a**, **c**: ×100; **b**, **d**: ×400)

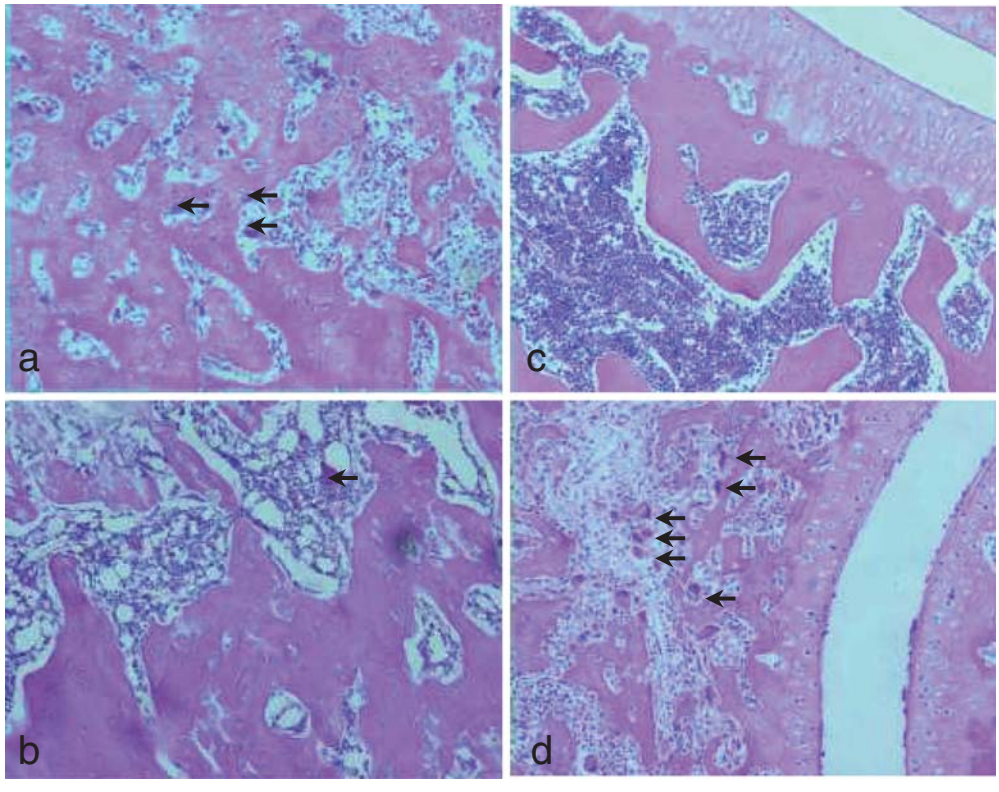

Fig. 5a–d. Histological findings at the proximal and distal tibial area in AA rats by treatment with etidronate and alendronate. **a**, **b** Histological findings at the proximal area by treatment with etidronate and alendronate, respectively. **c**, **d** Histological findings at the distal area by treatment with etidronate and alendronate, respectively. The large osteoclasts (*arrows*) at the proximal tibial area were preferentially reduced by treatment with alendronate (**b**), whereas it is the small osteoclasts at the distal tibial area that are preferentially reduced by etidronate (**c**), but these small osteoclasts (*arrows*) are not reduced by alendronate (**d**). (H&E and TRAP staining; **a**–**d** ×40)

Discussion

Rheumatoid arthritis represents an excellent model for gaining insights into the local and systemic effects of joint inflammation on skeletal tissues. Three forms of bone disease have been described in RA: focal bone loss affecting the immediate subchondral bone and bone at the joint margins, periarticular osteoporosis adjacent to inflamed joints, and generalized osteoporosis involving the axial and appendicular skeleton.[14,15]

Histological examination of periarticular osteoporosis adjacent to inflamed joints revealed the presence of local aggregates of inflammatory cells including macrophages and lymphocytes in the marrow space. There was an increase in the surface of bone covered by osteoids, as well as an increase in the resorption surfaces, which are often populated by osteoclasts.[16] Osteoclasts are multinuclear cells of the monocytic-macrophage lineage, and are generated from hematopoietic stem cells through a series of differentiation steps. The receptor activator of nuclear factor kappa B ligand (RANKL), expressed in osteoblasts, but also in synovial fibroblasts as well as T cells, is essential for the formation of osteoclasts.[17,18] Bisphosphonates are highly active as a cytotoxin for suppressing osteoclastic activity, and some are known to be clinically useful in pain relief in addition to suppression of bone resorption.[19,20] It has recently been observed that BPs exert an analgesic effect in AA rats and that etidronate was superior to alendronate in improving the pain threshold in these animals.[21,22]

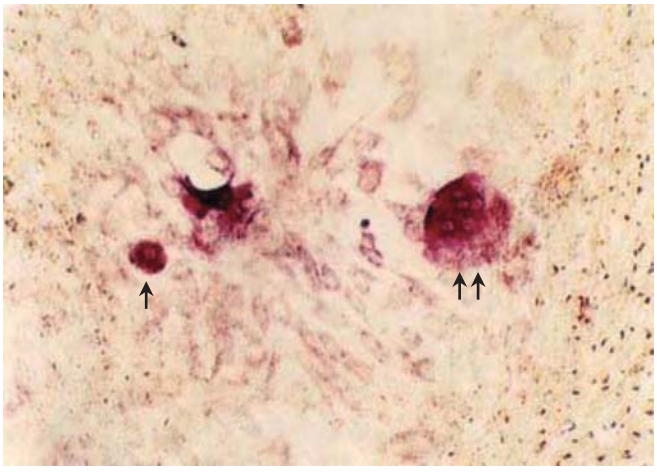

Fig. 6. Microscopic findings of tartrate resistant acid phosphatase positive cells in vitro. The *single arrow* shows a small osteoclast with less than four nuclei, and the *double arrows* show a large osteoclast with five or more nuclei. (TRAP staining, ×400)

Our results in AA rats showed that etidronate markedly improved BMD as measured by DEXA, not only in terms of the noninflammatory, proximal tibial area simulating systemic osteoporosis, but also the inflammatory, distal tibial area mimicking periarticular osteoporosis. Although alendronate improved the BMD of the proximal tibial area more than etidronate, the latter improved BMD of the inflammatory area more than the former. This suggests that etidronate acts more specifically than alendronate on the distal tibial area, which is adjacent to an inflamed ankle (or at least is the more inflamed site in this model), resulting in the suppression of bone destruction by inflammatory cytokines. It has been reported that etidronate more markedly reduces bone destruction than alendronate as assessed by soft X-rays in AA rats.[22] It was also reported that clodronate, a non-amino BP, shows anti-inflammatory ac-

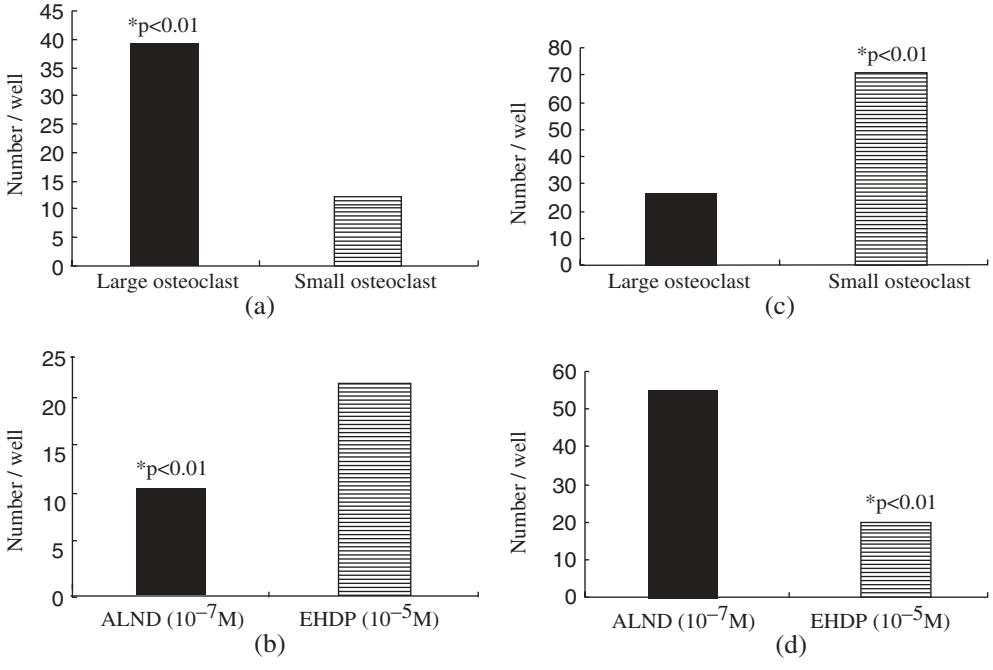

Fig. 7a–d. Osteoclast formation from the cells of the proximal tibial area and distal area. **a** A greater number of large compared to small osteoclasts is observed at the proximal tibial area ($P < 0.01$). **b** The alendronate (*ALND*; 10^{-7}M) group has significantly fewer large osteoclasts at the proximal tibial area compared to the etidronate (*EHDP*; 10^{-5}M) group ($P < 0.01$). **c** There is a greater number of small osteoclasts compared to large osteoclasts in the distal tibial area ($P < 0.01$). **d** The EHDP group has a significantly reduced number of small osteoclasts at the distal tibial area compared to the ALND group ($P < 0.01$)

tions in macrophage-like RAW 264 cells[23] and that it suppresses bone destruction in AA rats.[8] These results suggest that etidronate is more selectively incorporated into osteoclasts at periarticular sites than alendronate, thus exerting some anti-inflammatory effect on osteoclasts. On the other hand, it has been observed that incadronate, which is an amino BP, improves X-ray and histological findings, and paw volume, an indicator of inflammation, as well as reducing the number of osteoclasts in AA rats.[24]

Specific actions of BPs and their effects on cells other than osteoclasts are largely unknown. Nakamura et al.[25] suggested that non-amino BPs function via their suppressive effects on macrophages or granulocytes, whereas amino-type BPs exacerbate arthritis induced in mice; they concluded that this may be related to its ability to increase numbers of macrophages, granulocytes, and osteoclasts in the locality. However, both amino and non-amino type BPs inhibited physiological bone resorption, which is mediated by osteoclasts, at the proximal site of the tibia.[25] Particularly, intra-articular injection of clodronate leads to macrophage depletion and decreased expression of adhesion molecules, reflecting ongoing inflammation in the synovial lining in patients with RA.[26] Regarding the effects of amino-type BPs, Ralston et al.[27] and Tan et al.[28] have reported that they are not effective in restricting the progression of periarticular bone erosion, although bone resorption was suppressed.

In a clinical study of 40 RA patients, Ralston et al.[27] reported no improvement in markers of bone resorption or inflammation, or in X-ray findings following intravenous injection of pamidronate, an amino BP. In contrast, there is a report that oral administration of 40 mg alendronate reduces the plasma levels of interleukin (IL)-1, IL-6, and TNF-α as well as inhibiting bone resorption, and that its administration for >90 days also decreases erythrocyte sedimentation rate and C-reactive protein levels.[7] On the other hand, Dunn et al.[29,30] proposed that the anti-inflammatory action of non-amino type BPs may be related to the prevention of the development and subsequent recruitment of monocytes or macrophages. Thus it has been confirmed in clinical practice that non-amino and amino BPs possess anti-inflammatory actions, but differences in their actions on joint destruction in RA have not been reported so far. One study showed that etidronate suppresses infiltration of inflammatory cells into joints and bone marrow better than alendronate.[22] Furthermore, etidronate suppresses the production of prostaglandin E_2, substance P, and IL-6 in a dose-dependent manner at arthritis sites in AA rats.[31]

Taken together, our results document that the size and multinuclearity of osteoclasts and the number formed are different in the distal or proximal areas of the tibia, and that alendronate and etidronate mainly suppress different types of osteoclasts as discriminated by the number of nuclei. It is inferred from these observations that osteoclasts at the arthritic site and those at the proximal tibial area differ in their characteristics.

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