Original

Osteoclast Inhibitors for Bone Fracture Healing in Mice with High-Turnover Osteoporosis

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Abstract: The effectiveness of osteoclast inhibitors for high-turnover osteoporosis-related bone fracture is slightly unclear. This study aimed to examine the effectiveness of continuous administration of reveromycin A (RMA) and bisphosphonate (BP) in an osteoprotegerin knockout (OPG KO) model. The study included 8-week-old male OPG KO mice. The OPG KO mice received continuous saline (OPG SA group, n = 4), BP (OPG BP group, n = 4), or RMA (OPG RMA group, n = 4). The study also included wild-type mice as controls (administered saline; Wild SA group, n = 4). We found that serum alkaline phosphatase activation (parameter for full-body bone formation) was significantly higher in the OPG SA group than in the Wild SA, OPG BP, and OPG RMA groups and was significantly lower in the OPG BP group than in the OPG RMA group (all p < 0.001). Additionally, the blood tartrate-resistant acid phosphatase concentration (parameter for osteoclast activation) was significantly higher in the OPG SA group than in the Wild SA, OPG BP, and OPG RMA groups and was significantly lower in the OPG BP group than in the OPG RMA group (all p < 0.001). Moreover, at 21 days, the score was significantly lower (p < 0.01) and the callus area was significantly larger (p < 0.001) in the OPG BP group than in the OPG SA and OPG RMA groups. In the OPG RMA group, the fractured bone segment was indistinguishable from the rest of the bone at 21 days. Our results suggest that RMA is an effective and safe alternative to BP for the treatment of high-turnover osteoporosis.

Key words: Bone fracture, Osteoclasts, Osteoporosis, Osteoprotegerin, Reveromycin A

Introduction

Osteoporosis can be divided into two different types3-6): low-turnover osteoporosis, which is primarily due to age-related decreases in bone formation; and high-turnover osteoporosis, which is primarily due to accelerated bone absorption due to factors such as menopause. Patients with osteoporosis regularly use osteoclast inhibitors like bisphosphonates (BPs) as preventative treatment, in order to decrease the serious risk factors of bone fracture3-6). However, reports have indicated that these drugs have long-term side effects, such as jaw-bone necrosis or bone fracture due to the decreased bone metabolism7). BPs are deposited in the bone matrix and taken up by the osteoclast. They have a long half-life, and side effects due to their accumulation in bone tissue, including jaw-bone necrosis, have been reported3-5). In order to resolve these issues, numerous drugs, such as denosumab8-13), have been developed in recent years. Reveromycin A (RMA) is one of these recently developed drugs. RMA is a polyketide isolated from the actinomycete Streptomyces sp. and has a remarkably short half-life. It is taken up only by activated osteoclasts that have bone absorption capabilities, and, thus, not by inactive osteoclasts or osteoclast precursor cells, and disrupts bone absorption by inhibiting protein synthesis and inducing apoptosis14). Another important characteristic of RMA is that it is not deposited within the bone14). Moreover, its side effects are thought to be minor, based on its specific uptake by active osteoclasts in an acid environment and its short half-life15).

Osteoclast formation and bone absorption were shown to be accelerated in osteoprotegerin knockout (OPG KO) mice16). Reports have demonstrated that although the bones of these mice are normal at birth, bone loss starts in the trabecular bone one week after birth and in the cortical bone, which is primarily composed of woven bone, four weeks after birth. Osteoclast activation is accelerated with mouse growth, and mature OPG KO mice have thin/lower-density trabecular bones and serious, high-turnover osteoporosis compared to their wild-type littermates17,18). Additionally, experimental treatments for bone fracture in OPG KO mice by Ota et al. showed that the bone fracture segments join together more rapidly in OPG KO mice than in their wild-type littermates, since OPG acts as a negative regulator of cartilage-cell formation19).

Therefore, our objective was to create an experimental, bone-fracture model using OPG KO mice and examine the effectiveness of continuous administration of RMA and BP on high-turnover osteoporosis-related bone fracture.

Materials and Methods

Animals, surgery, and test drugs

We used 8-week-old male OPG KO mice bred at the Animal Laboratory in the School of Dentistry, Aichi Gakuin University, Japan. Animals were kept at 22 ± 2°C room temperature, 50 ± 10% relative humid-
ity, and an illumination period cycling every 12 hours. Solid feed CE-2 (CLEA Japan Inc., Tokyo, Japan) and tap water were provided ad libitum. The management of research animals and all research methods were approved by and performed according to the guidelines of the Aichi Gakuin University School of Dentistry Animal Experiment Committee (Approval number AGUD227).

OPG KO mice were divided in three test groups: a continuous saline-administered group (henceforth OPG SA group, n = 4), a continuous BP-administered group (henceforth OPG BP group, n = 4), and an RMA-administered group (henceforth OPG RMA group, n = 4). Wild-type mice administered with saline (henceforth Wild SA group) were used as a control group.

To generate the experimental bone fracture model, mice were anesthetized using a mixture of three types of anesthetic agents (medetomidine hydrochloride, midazolam, butorphanol tartrate) and a closed transverse fracture was induced in the tibia of mice in each group. Prior to all treatments, the section below the knee, in the back foot of the mouse, was shaved, and a vertical incision was made in the outside of the knee joint. The bone marrow in the boundary between the upper end of the tibia and knee-cap was exposed using a 0.5-mm diameter steel bar (Dentsply Sirona Inc., Tokyo, Japan). Then, a 0.016-inch (0.4064-mm) diameter, ~1.5-cm long β-Ti wire (KaVo Dental Systems Co., Ltd., Japan, Tokyo, Japan) was inserted in the tibia marrow as an intramedullary rod, and the wound was closed. Thereafter, force was applied to the central tibia area using an HSL 423-12 three-jaw plier (Hammacher Instrumente, Solingen, Germany). The minimal amount of force necessary to induce bone fracture was applied, and fracture was confirmed by feeling the bone with fingers. Then, the tibia was sampled by taking the entire femoral region 10 and 21 days after the procedure, based on methods outlined by Ota et al.

The test drugs used were RMA 3Na salt (RIKEN CSRS, Saitama, Japan) for RMA and alendronate (Teiroc®, Teijin Pharma Co., Ltd., Tokyo, Japan) for BP. Intraperitoneal administrations of RMA (1.0 mg/kg of weight/twice per day; based on Tanaka et al.) and alendronate (1.25 mg/kg of weight; based on Tabuchi et al.) were conducted once a day immediately after bone fracture was induced, for a total period of three weeks.

Bone metabolism marker measurements in the serum

Blood samples were collected from the mice 21 days after the procedure under anesthesia using a mixture of three different types of anesthetics (medetomidine hydrochloride, midazolam, butorphanol tartrate). After the samples were collected, they were centrifuged at 3,000 rpm for 6 minutes to collect the serum. Serum alkaline phosphatase (ALP) activation was measured using Liquitech ALP (Roche Diagnostic K. K., Tokyo, Japan). Additionally, the concentration of tartrate-resistant acid phosphatase (TRAP) in the blood was measured using an ELISA kit (Immundiagnostic Systems Ltd., Ontario, Canada).

Soft X-ray imaging

We used the OMC-403 soft X-ray apparatus (OHMIC Co., Ltd., Shiga, Japan) at a voltage of 23 kV, current of 3 mA, and exposure time of 3 s using ISO speed D X-ray dental film DIK-10 (Hanshin Technical Laboratory Ltd., Hyogo, Japan).

Film development and fixing were performed using a GBX developer and the TWIN PACK fixer (Kodak Ltd., Tokyo, Japan). Soft X-ray images were digitally scanned at a resolution of 1,200 dpi using an office GS-10000G image scanner (EPSON Co., Ltd., Tokyo, Japan). The soft callus-formation area on X-ray images at 10 and 21 days was calculated using ImageJ64 image analysis software. The area of the newly formed callus was measured by referencing a report by Warden et al.
Due to the differences in film contrast between samples that arose during processing, we made the background color uniform and then arbitrarily optimized the image contrast to make it easy to distinguish the outline of the callus. We paid particular attention to adjusting the images so the original periosteum present on the tibial surface before the fracture could be identified. In addition, the same image was used to evaluate the fracture healing score by referencing the report by Smitham et al.26 (Table 1). Regarding scores 1 and 2, a score of 1 was given when the thickness of the callus was ≤50% of the long axis of a horizontal section of the cortical bone at fracture, and a score of 2 was given for thicknesses >50%. To prevent evaluation bias, the evaluations were performed by 3 evaluators who were blind to which experimental group the samples belonged to, and the samples were evaluated in random order.

**Histopathological observations**

The sampled tibia was fixed in a 10% neutral-buffered formalin solution for a week and then decalcified in 10% EDTA (pH 7.2) at 4°C for four weeks. The samples were embedded in paraffin and sectioned...
serially at 5 μm in the sagittal plane. Sections were stained with hematoxylin-eosin and toluidine blue (t-blue) and examined under an optical microscope.

**Statistical analysis**

Collected experimental data are expressed as mean and standard deviation. Statistical significance tests were performed using Tukey’s multiple comparison test. GraphPad Prism v. 6 (GraphPad Software Inc., San Diego, CA, USA) was used for all statistical analysis. Statistical significance was set at p < 0.05.
Results
Bone metabolism markers in the serum
Serum ALP activation, a parameter indicative of full-body bone formation, was statistically significantly higher in the OPG SA group than in the Wild SA, OPG BP, and OPG RMA groups. Moreover, statistically significantly lower ALP activation levels were found in the OPG BP than the OPG RMA group (p < 0.001; Fig. 2A).

Additionally, blood TRAP concentration, a parameter for osteoclast activation, was statistically significantly higher in the OPG SA than the Wild SA, OPG BP, and OPG RMA groups but significantly lower in the OPG BP than OPG RMA group (p < 0.001; Fig. 2B).

Soft X-ray image evaluation
Comparison of bone fracture treatment scores
Statistically significant increases in bone healing score were observed from 10 to 21 days following the procedure in the OPG SA and OPG RMA groups (p < 0.001; Fig. 3A, B). In contrast, no statistically significant differences were observed among the different drug treatments at 10 days following the procedure, fracture treatment score was significantly lower at 21 days following the procedure in the OPG BP than the OPG SA and OPG RMA groups (p < 0.01; Fig. 3A, B).

Effects on callus area
Statistically significant decreases in callus area were observed from 10 to 21 days following the procedure in the OPG SA and OPG RMA groups (p < 0.001; Fig. 3A, C), whereas no significant differences were observed in the OPG BP group.

Although no statistically significant differences were observed among the drug-treatment groups at 10 days following the procedure, the callus area at 21 days following the procedure was statistically significantly larger in the OPG BP than in the OPG SA and OPG RMA groups (p < 0.001; Fig. 3A, C). No statistically significant differences were observed in the OPG RMA and OPG SA groups.

Histological observation of the callus
Histological examination of the callus indicated the formation of cartilage, connective tissue, and numerous osteoids in the fractured bone segments of the OPG SA group at 10 days following the procedure, while crosslinks to join the fractured cortical bone also began to form at this timepoint. Crosslinks of the cortical bone tissue with a lamellar structure were forming at 21 days following the procedure, and we could no longer observe the line of fracture at this point (Fig. 4).

Abundant hyaline cartilage formation was observed in the OPG BP group at 10 days following the procedure. Additionally, cartilage-like tissue persisted at 21 days after the procedure, with osteoids surrounding this tissue, and crosslinks forming to link the fractured bone stumps. The presence of cartilage-like tissue was also confirmed with t-blue staining.

In the OPG RMA group, cartilage and connective tissue had formed at the fractured bone segment at 10 days following fracture, and the presence of cartilage cells was also confirmed with t-blue staining. Crosslinks due to lamellar-structure cortical bone tissues were forming at 21 days following the procedure, the line of fracture was no longer visible, and the fractured bone segment was indistinguishable from the rest of the bone.

Bone fracture healing and OPG KO mice
Bone fracture healing is generally divided into four phases: an inflammatory, a tissue growth, a callus formation, and a bone remodeling phase. Histologically, during the inflammatory phase, blood hematoma is formed at the fractured bone segment, while inflammatory tissues seep into the hematoma, and necrotic tissue is absorbed. During the soft callus phase, the blood hematoma is replaced by granulation tissue, and soft calluses are formed from cartilage that carries small amounts of bone. During the hard callus formation phase that follows, the formed cartilage is replaced by fibrous calluses via calcification, and crosslinks are formed across the fractured bone segment. Although this fibrous callus is composed of an undeveloped cancellous bone-like structure, this is replaced by a well-developed lamellar bone during the remodeling phase, and the bone returns to its pre-fracture form.

Osteoblasts and osteoclasts are both involved in these four phases and are thought to contribute to the completion of endochondral ossification required for bone fracture healing. Osteoclasts constitute the only bone cell type that destroys and absorbs the calcified bone matrix; their differentiation, maturation, and functioning are tightly regulated by receptor activator of nuclear factor-κB ligand (RANKL), which is located on the cell membrane of osteoclasts or bone marrow stromal cells. In particular, osteoclasts and their precursor cells express RANK (a RANKL receptor), recognize RANKL via intercellular contact, and differentiate into mature osteoclasts. Meanwhile, OPG produced by osteoblasts belongs to the tumor necrosis factor receptor superfamily and is a decoy RANKL receptor. OPG controls osteoclast differentiation and functional expression by strongly controlling RANKL and RANK interaction.

Serious high-turnover osteoporosis is considered to occur in OPG KO mice due to the acceleration of osteoclast formation and bone absorption. However, in our experiments, tissue imaging showed woven bone-like cortical bones and sparse cancellous bone structure in the non-fractured bone segments. Human juvenile Paget’s disease, which affects the bones, is thought to be active in OPG KO mice and is characterized by an extremely high, bone, metabolic turnover, with symptoms observed at young age, including progressive bone abnormalities in the skeletal frame, severe osteoporosis, accompanying bone fractures, and decreased height.

Additionally, ALP activation and soluble RANKL are thought to increase when OPG is genetically ablated. Consistently, our experiments also showed that both markers of bone metabolism were higher in the OPG SA group than in the Wild SA group, and an accelerated bone metabolic turnover was observed.

Osteoclast inhibitor effects
Although no statistically significant differences were observed between the OPG RMA and OPG SA groups in terms of the X-ray score and callus area, histologically, the OPG RMA group had a higher amount of residual cartilage tissue than the OPG SA group at 10 days following the procedure. Afterwards, there were no statistically significant differences between the two groups at 21 days following the procedure. In contrast, the OPG BP group had higher callus formation than the OPG SA group in terms of bone healing score, callus area, and histology at 10 days following the procedure; this higher extent of bone formation was maintained, and the continued presence of the line of fracture was confirmed at 21 days following the procedure. The cartilage tissue that was histologically confirmed to be abundantly present at 10 days was still residually present at 21 days following the procedure.
Based on these results, we conclude that the decreased callus presence observed during the standard bone healing process does not occur in the OPG BP group, and that healing is consequently delayed.

BP is currently the drug of choice for osteoporosis. It is a synthetic pyrophosphate analogue that directly acts on osteoclasts. It functions by inducing apoptosis\textsuperscript{36,37} and controlling bone absorption\textsuperscript{38,39}. Consistently, we found that cartilage tissue growth and differentiation became relatively dominant as a result of osteoclast activation after BP administration, resulting in increased cartilage presence. However, the bone matrix structure remained undeveloped, since the cartilage was not absorbed afterwards, which is thought to prevent the typical progress of endochondral ossification during bone fracture healing.

A secondary effect of BP is improved bone density; however, this can result in atypical fractures due to the bone becoming hard and brittle\textsuperscript{40,41}. In other fields, like dentistry, studies have indicated that BP administration can result in jaw bone necrosis during tooth extraction\textsuperscript{42,43}. As such, there are numerous problems with BP administration, despite it being the drug of choice for diseases such as osteoporosis or juvenile Paget’s disease of the bone\textsuperscript{44}.

Mashiba et al.\textsuperscript{45} reported that the risk of bone fracture is limited in patients with osteoporosis who are administered BP, and that the marked increase in calluses at the fractured bone segment due to BP increases the strength of the fractured bone region. However, the authors also reported that callus maturation is delayed due to the strong controls imposed by callus remodeling, and that delays in the process of bone fracture healing are compensated by the callus amount\textsuperscript{46}. In our study, BP administration also inhibited cartilage absorption at all phases of bone fracture healing.

Regarding RMA, Woo et al.\textsuperscript{17} reported that it has high tissue selectivity, does not remain in the bone matrix, and acts only on activated osteoclasts in an acidic environment. In addition, we have administered RMA as a treatment for periodontal disease in past studies\textsuperscript{47} and have reported its high cell selectivity and usefulness. Furthermore, its half-life is short. Accordingly, as evidenced by our serum sampling analysis and histology, RMA exerted a strong inflammatory response while still controlling activated osteoclasts. Although it behaved as if it delayed the process of bone fracture healing in the early stages of the process, during which many activated osteoclasts appear, the extent of healing in the late stages was roughly the same as that of the saline-treated OPG SA group, while none of the inhibitory effects on bone fracture healing that were observed with BP administration were noted with RMA.

Further work, including testing the strength of the fractured bone segment, is necessary to confirm the present findings. Nevertheless, our study indicates the potential of RMA as an alternative treatment in the place of BP for osteoporosis and juvenile Paget’s disease of the bone.

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