FEATURE ARTICLES

Isotope microscopic assessment for localization of ^{15}N-minodeonate in bone

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ABSTRACT : Minodronate has been highlighted for its sustained effects on osteoporotic treatment. To determine the cellular mechanism of its sustained effects, we have assessed the localization of minodronate in mouse bones through isotope microscopy, by labeling it with a stable and rare nitrogen isotope (^{15}N-minodronate). Eight-weeks-old male mice intravenously received ^{15}N-minodronate (1 mg/kg) were fixed after three hours, 24 hrs, one week and one month. Isotope microscopy localized ^{15}N-minodronate predominantly beneath osteoblasts (bone forming surface) rather than nearby osteoclasts (bone-resorbing surface). Literally, alendronate, another nitrogen-containing bisphosphonate, has been reported to accumulate on the bone-resorbing surface, and suddenly inhibit the osteoclasts. In contrast, minodronate appears to coat the bone-forming surface, without immediate inhibition of osteoclasts. A single injection of minodronate chronologically increased metaphyseal trabeculae, whereas the numbers of tartrate resistant acid phosphatase (TRAP)-positive osteoclasts and alkaline phosphatase (ALP)-reactive osteoblastic area were not reduced. Apoptotic osteoclasts were not apparent, but, finally being observed in the later stage of the experiments, while ALP-reactive osteoblasts were persisted on the trabeculae. Osteoclasts have developed ruffled borders at 3 hrs after minodronate administration; however, osteoclasts were roughly attached to the bone surfaces and did not form ruffled borders at 24 hrs after the administration. Von Kossa staining clearly demonstrated that osteoclasts did not incorporate the minodronate-treated bone matrix, while osteoclasts included abundant bone minerals inside in the control specimens. Taken together, minodronate accumulates in bone underneath osteoblasts rather than under bone-resorbing osteoclasts ; therefore, it is likely that the osteoclasts are not able to resorb and incorporate the minodronate-coated bone matrix, which may result in osteoclastic survival, avoiding osteoclastic apoptosis and consequently inducing cell coupling with osteoblasts. In conclusion, the resistance of miniodronate-coating bone from osteoclastic resorption, and the consequent cell coupling with osteoblasts appear to produce a long-lasting and bone-preserving effect.

Key Words : *minodronate, isotope microscopy, cell coupling, osteoclast, osteoblast*

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Ⅰ. Introduction

In a past scare, bisphosphonates have been positioned as mainstream drugs for the osteoporotic treatment. Drugs of this class have high affinity for crystalline calcium phosphates[1-3], thus binding to the mineralized bone matrix when patients take this medicine. When osteoclasts degrade bone minerals bound to bisphosphonates, bone resorption is inhibited as the drug finally induces osteoclast apoptosis[4, 5].

Among the several commercially available bisphosphonates, alendronate is the most commonly used one. In general, nitrogen-containing bisphosphonates – pamidronate, alendronate, ibandronate, zoledronate and risedronate, act by inhibiting farnesyl pyrophosphate synthase, an enzyme involved in the mevalonate pathway[6, 7], which then impairs protein prenylation of the small GTPase of the Ras family[8]. These small GTPase proteins play crucial roles for vesicular trafficking and cytoskeletal organization of bone-resorbing osteoclasts, and finally cell survival[9, 10] ; Nitrogen-containing bisphosphonates force osteoclasts to cease from resorbing bone mostly due to disrupted vesicular trafficking and cytoskeleton misassemble[11].

Alternatively, bone formation is coupled with bone resorption during bone remodeling, which continuously takes place along the bone surfaces with resorption-preceding formation. There are many evidence supporting the hypothesis that osteoclastic bone resorption would trigger the differentiation and activation of osteoblasts, – a process referred to as a "coupling phenomenon"[12-15]. Without osteoclasts, indeed, osteoblastic population, osteoblastic bone formation and bone mineralization are markedly diminished, for instance, in *op/op* mice[16, 17]. One of our studies, in addition, demonstrated that cell coupling between osteoclasts and preosteoblasts must take place for the parathyroid hormone (PTH)-driven bone anabolic effect to occur[18]. Therefore, the drop in osteoclastic bone resorption induced by bisphosphonate administration may cause a reduction both in osteoblastic population and in bone formation.

Minodronate, a third-generation, nitrogen-containing bisphosphonate, is an approved drug for the osteoporotic treatment in Japan[19-21], and has been shown to suppress bone resorption in ovariectomized rats and *Macaca Fascicularis*[22-24], and affected cortical bone response to mechanical loading in rats[25]. In addition, other *in vivo* and *in vitro* studies reported on the high potency of minodronate compared to alendronate regarding the inhibition of bone resorption[26], with an intermediate mineral-binding affinity[27].

Since osteoporotic patients seem to prefer weekly and monthly dosing regimens[28], long-acting bisphosphonates such as minodronate are gradually becoming the first line of osteoporotic treatment for prescribing physicians. Still, a pervasive question among medical professionals and researchers is how can a single dose of bisphosphonate sustain its effects for over a month? Therefore, we assumed that the localization of minodronate may provide a clue that might help address this issue. In order to do so, the use of radioisotope-labeling may strongly assist on determining the localization and distribution of minodronate *in vivo* ; however, animal studies involving radioisotopes are ethically questionable and therefore, currently restricted. To circumvent this limitation, we have thought up to use an isotope microscopy instead – a mode of secondary ion mass spectrometry and two-dimensional isotope detection technique[29-31]. Recently, isotope microscope systems have been used to analyze living matter specimens[32]. For assessing the localization of minodronate, in our study, ^{15}N of minodronate molecule was substituted with ^{14}N. The ^{15}N-minodronate was then injected into mice to determine the localization of ^{15}N-minodronate through using isotope microscopy.

In this min-review, we will introduce our recent investigation on the localization of minodronate in murine bone, and behavior of bone cells after the minodronate administration[33].

Ⅱ. Isotope microscopy and design of animal experiment

Isotopes are powerful tracers to determine origin and circulation of elements in nature, and decoding of isotopic zonings of minerals is useful to study historical environmental variations. Recently, in the biological research field, isotope microscopy has been applied to assess the localization of isotopes of the elements in mineralized tissues, such as bone[33], dentin[34] and shell[35].

In our own study, ^{14}N and ^{12}C in the minodronate molecule were substituted with the stables isotopes ^{15}N and ^{13}C (both are rare in nature) to generate {1-hydroxy-2-[(1-^{15}N)imidazo[1,2-a]pyridin-3-yl]($^{13}C2$)ethane-1,1-diyl} bis(phosphonic acid) hydrate. The isotope microscope located at the Creative Research Institution, Hokkaido University[29] was used for identification and localization of ^{15}N of ^{15}N- and ^{13}C-labelled minodronate (^{15}N-minodronate).

Eight-weeks-old male ICR mice were anesthetized, and then, were injected with ^{15}N-minodronate (1 mg/kg) through the external jugular vein. Three hours, 24 hrs, 1 week and 1month after the injection, femora and tibiae were extracted and embedded in paraffin and epoxy resin. Isotope microscope system located in Hokkaido University was used to visualize the distribution of ^{15}N-minodronate in the bone tissue, a technique known as isotopography[29-35].

Ⅲ. Minodronate is predominantly localized on bone-forming surface

At early 3 hrs after minodronate administration, most trabecular surfaces showed traces of ^{15}N-minodronate with varying intensities of ^{15}N-minodronate labeling (Fig. 1). After 1 month, ^{15}N-minodronate labeling was seen only on trabeculae that were distant from the growth plate cartilage (Fig. 2). However, isotope microscopic images at 3 hrs after minodronate injection showed a faint ^{15}N-minodronate labeling underneath osteoclasts, while an intense labeling of minodronate was seen adjacent to mature, cuboidal osteoblasts (Fig. 3a-d). The frequency histogram for osteoblast and osteoclast numbers and the ^{15}N/^{14}N intensity ratio demonstrated that most osteoclasts were found on bone surfaces with low ^{15}N/^{14}N ratios, while many osteoblasts were seen on bone surfaces with varying ^{15}N/^{14}N intensity ratios (Fig. 3e).

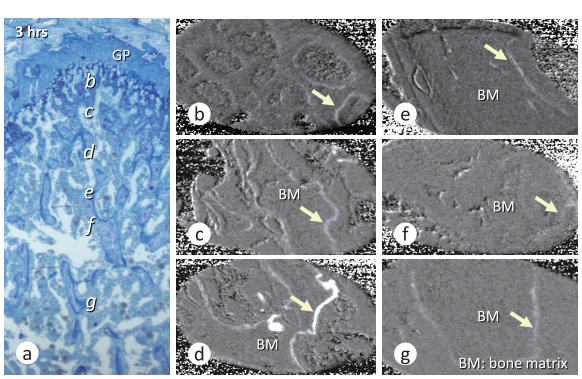

Fig. 1 *Isotope microscopic images of ^{15}N-minodronate localization at 3 hrs after the injection*

Panels a-g show the localization of ^{15}N-minodronate at 3 hrs after minodronate administration. Panel a shows a semi-thin section stained with toluidine blue, which represent the area of isotope microscopy observation. At 3 hrs, all the trabecular surfaces showed white lines indicative of ^{15}N-minodronate in all the areas (yellow colored arrows, b-g).
meta : metaphysis, GP : growth plate, BM : bone marrow
Modified images of Hongo et al.[33] with permission

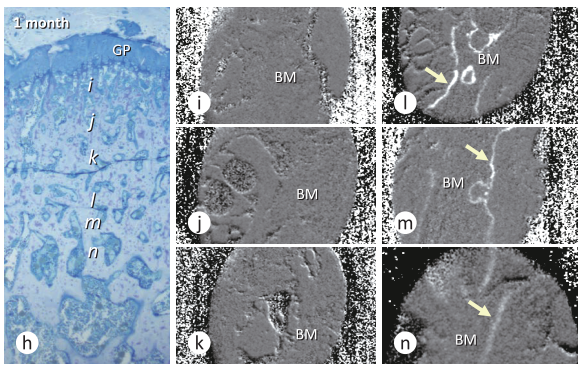

Fig. 2 *Isotope microscopic images of ^{15}N-minodronate localization at 1 month after the injection*

Panels h-n show the localization of ^{15}N-minodronate at 1 month after minodronate administration. Panel h shows a semi-thin section stained with toluidine blue, which represent the area of isotope microscopy observation. After 1 month later, the regions close to the growth plate do not show the labeling of ^{15}N-minodronate, though the distant regions revealed ^{15}N-minodronate (See white lines indicated by yellow arrows in l-n).
meta : metaphysis, GP : growth plate, BM : bone marrow
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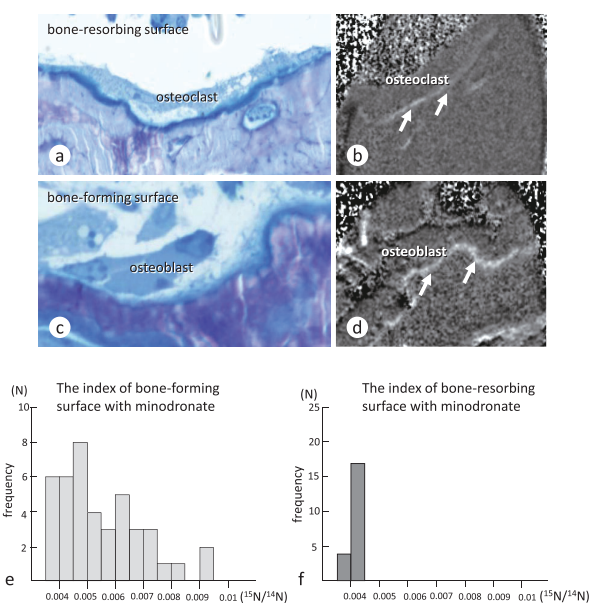

Fig. 3 *Localization of ^{15}N-minodronate on bone-forming surface and bone-resorbing surface*

Panels a and c are serial semi-thin sections stained with toluidine blue, while panels b and d are isotope microscopic images at 3 hrs after minodronate injection. Note a faint ^{15}N-minodronate labeling underneath osteoclasts (a, b), while an intense labeling of minodronate adjacent to mature osteoblasts (c, d). Panels e and f are the histogram demonstrating the number of osteoblasts (bone-forming surfaces) and osteoclasts (bone-resorbing surfaces) located on a regions of varying ^{15}N/^{14}N intensity ratios.
bm : bone marrow
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Thus, isotope microscopy clearly demonstrated ^{15}N-minodronate mainly underneath osteoblasts, *i.e.*, on bone formation surfaces, rather than near bone-resorbing osteoclasts. In contrast, an autoradiographic study showed that alendronate accumulated on resorption surfaces[36], which suggests that alendronate would be readily incorporated by bone-resorbing osteoclasts and immediately halt bone resorption. Therefore, alendronate seems to target bone-resorbing osteoclasts directly, while minodronate's distribution and localization on bone formation surfaces indicate that this drug does not target bone-resorbing osteoclasts, but instead, appears to accumulate on the bone matrix beneath mature osteoblasts.

Ⅳ. Minodronate increases metaphyseal bone volume without affecting the osteoclasts' localization

Femora treated with minodronate showed chronological increases of metaphyseal bone (Fig. 4a-d). Trabeculae were more abundant at later time points (1 week and 1 month) than at earlier time points (3 hrs and 24 hrs) after the injections, consistent with an elevated index of bone volume/tissue volume (data not shown). Therefore, a single injection of minodronate seems to produce a long-lasting effect for increasing bone volume.

Interestingly, the distribution of tartrate resistant acid phosphatase (TRAP)-positive osteoclasts and alkaline phosphatase (ALP)-reactive osteoblasts[37, 38] was very similar compared with those of the control specimens in all the experimental periods (Fig. 4e-h), although other bisphosphates apparently affect the osteoclast numbers[39]. Apoptotic osteoclasts began to appear in 1 week and 1 month samples, but several osteoclasts could still be present on the trabecular surfaces (Fig. 5). Consistent with less numbers of apoptotic osteoclasts, ALP-reactive osteoblasts seemed to be persistent though it slightly tended to be decreased. In addition, osteocytes seemed intact, and no signs of atrophy or microdamage were identified at all time points, despite the presence of many atrophied osteocytes in a regimen of daily administration of alendronate[39].

When observing under transmission electron microscopy, at 3 hrs after minodronate administration, osteoclasts showed typical ruffled borders adjacent to the underlying bone surfaces, while cuboidal, mature osteoblasts were lying on the trabecular surfaces.

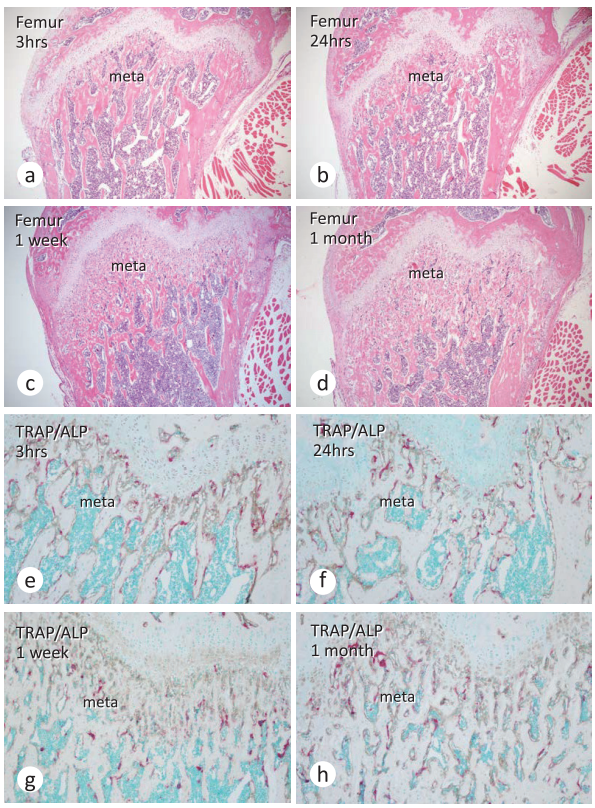

Fig. 4 *Chronological changes of femoral trabeculae and the distribution of TRAP-positive osteoclasts and ALP-reactive osteoblasts after minodronate administration*

Femora treated with single administration of minodronate increased metaphyseal trabeculae as time goes on. Note many trabeculae at 1 week (c) and 1 month (d) when compared with those at 3 hrs (a) and 24 hrs (b) after injection. The distribution of TRAP-positive osteoclasts (red color) and ALP-reactive osteoblasts (brown color) is similar at all the time points (e-h). Note there seems no significant differences in the numbers of TRAP-positive osteoclasts in all the experimental periods.
meta : metaphysis
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However, 24 hrs after minodronate injection, osteoclasts were partially detached from the bone surfaces and had lost their ruffled borders.

Taken together, a single administration of minodronate increased metaphyseal trabeculae, without reducing the numbers of osteoclasts and osteoblasts, and without promoting apoptotic osteoclasts at the early time points. The most important finding is that osteoclasts were not damaged, and did not develop the ruffled borders after the minidronate administration. This means that osteoclasts cannot resorb the minodronate-treated bone matrix because of no ruffled borders ; however, such osteoclasts seems to be enough to induce surrounding osteoblasts to an active form, *i.e.*, mature osteoblasts. This implies cell coupling between osteoclasts and

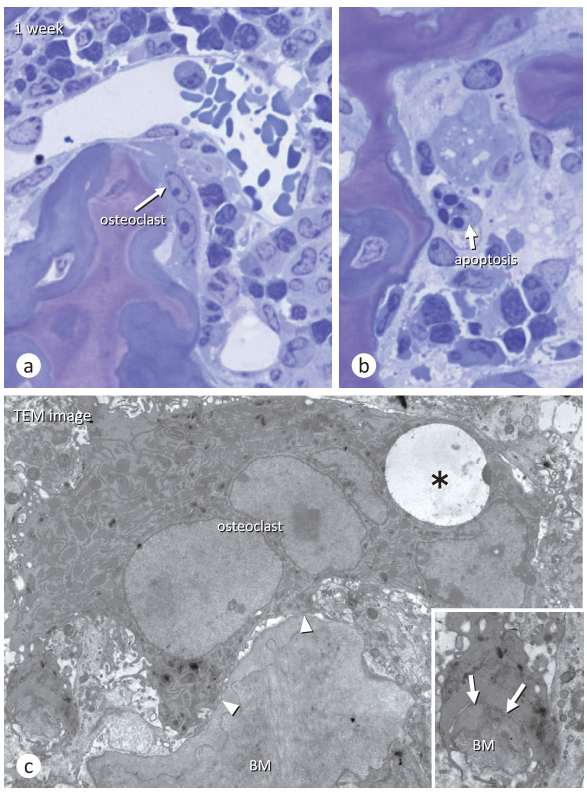

Fig. 5 *Osteoclasts at 1 week after minodronate administration*
Toluidine blue staining of semi-thin sections (a, b) shows that some osteoclasts are partially attached to the bone surfaces despite the lack of ruffled borders (an arrow, a), and several are apoptotic (double arrows, b) at 1 week after minodronate administration. Transmission electron microscopic (TEM) observation demonstrates osteoclasts with collapsed nuclei (an asterisk) and a lack of ruffled borders (See arrowheads, c). Such defective osteoclasts extended short cytoplasmic processes towards the bone matrix (BM, arrows, an inset of c).
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osteoblasts in minodronate-treated bone. Another advantage of minodronate seems to be the presence of normal osteocytes. Contrary, the daily administration of alendronate induced the osteocytic atrophy and discontinuous connectivity of osteocytic processes, indicating disrupted syncytia of osteocytic network[39]. Taken together, a single injection of minodronate seems to produce a long-lasting effect with keeping cell coupling between osteoclasts and osteoblasts, as well as maintaining functional syncytia of osteocytes.

Ⅴ. How does minodronate affect osteoclasts?

However, one may wonder why and how minodronate could increase bone volume without reducing the localization and the numbers of osteoclasts. In order to clarify this issue, we have employed von Kossa staining to verify if osteoclasts are able to resorb and incorporate the mindronate-treated bone matrix (Fig. 6). As a consequence, von Kossa staining showed osteoclasts including mineralized bone matrix inside in the control group, while osteoclasts failed to incorporate the minodronate-treated bone minerals (Compare Figs. 6b and c).

Taking all findings into consideration, it is inferred that osteoclasts are not capable of resorbing minodronate-coated bone. In other words, the bone-forming surfaces are coated with minodronate as shown by isotope microscopy, and osteoclasts may fail to resorb the minodronate-coated bone matrix. If osteoclasts do not degrade the minodronate-coated bone matrix, they would not be exposed to minodronate and would not, therefore, enter apoptosis – at least immediately upon administration of the drug. Hence, a single injection of minodronate seems to produce a long-lasting effect that avoids osteoclastic bone resorption by coating the bone surface and rendering it somewhat "resorption-proof". As mentioned above, the dysfunctional, non-resorbing osteoclasts may allow nearby osteoblasts somehow to be activated through cell coupling.

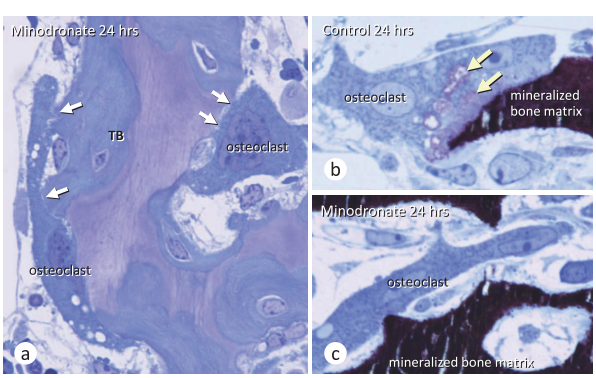

Fig. 6 *Minodronate-treated osteoclasts at 24 hrs*
Panels a-c show semi-thin sections stained with toluidine blue. At 24 hrs after minodronate injection, osteoclasts are shown to be partially detached from the bone surfaces without their ruffled borders (white arrows, a). Von Kossa staining visualizes that control osteoclasts without minodronate treatment incorporate fragments of mineralized bone matrix (brown color, yellow arrows, b). However, after minodronate administration, no osteoclasts are shown to engulf mineralized bone matrices inside (c).
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Ⅵ. Perspective

Retaining osteoblastic activity may favor combined therapy with an anabolic drug such as teriparatide, hPTH(1-34)[40]. Daily alendronate administration appears

to reduce the anabolic effect of hPTH(1-34) on hip bone mineral density and cortical volume[41, 42]. Therefore, if minodronate sustains osteoblastic activities, as suggested by our findings, it could also produce better results when combined with teriparatide. In fact, one recent study described that a combination of minodronate and teriparatide resulted in increased bone volume and trabecular number, while reducing trabecular separation compared with teriparatide alone[23]. Additionally, another advantage seems to be less damages of osteocytes after a single administration of minodronate. Osteocytic network is involved in bone quality, *that is*, sensing the degree and direction of mechanical stress, molecular transport through osteocytic cytoplasmic processes and osteocytic canaliculi, regulation of bone remodeling mediated by sclerostin and controlling of serum concentration of phosphate by secreting fibroblast growth factor 23[43-46]. Taken together, we hypothesize that the single injection regimen of minodronate and its localization on bone-forming surface appear to provide several benefits for osteoporotic treatment.

Ⅶ. Conclusion

In conclusion, minodronate accumulates in bone underneath osteoblasts rather than under bone-resorbing osteoclasts ; therefore, it is likely that the minodronate-coated bone matrix is resistant to osteoclastic resorption, which results in a long-lasting and bone-preserving effect.

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