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Forum Minireview

Current Topics in Pharmacological Research on Bone Metabolism: Inhibitory Effects of Bisphosphonates on the Differentiation and Activity of Osteoclasts

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Abstract. Despite the extensive use of bisphosphonates (BPs) in the treatment of metabolic bone diseases associated with increased osteoclastic bone resorption, the precise mechanism of their action on bone metabolism is still unclear. To clarify at which stages of osteoclast differentiation and activation that BPs influence, we examined the osteoclasts generated from mononuclear precursors and osteoclasts in the calvaria by laser scanning confocal microscopy. The studies showed that BPs inhibit lipopolysaccharide- or parathyroid hormone-induced osteoclast differentiation, fusion, attachment, actin ring formation, and activation and that both β3 integrin and osteopontin have an important role in cytoskeletal rearrangements associated with cell attachment and resorption in osteoclasts.

Keywords: bisphosphonate, osteoclast, calvaria, confocal microscopy, osteopontin

Introduction

Bisphosphonates (BPs) have a similar chemical structure to that of inorganic pyrophosphate and bind strongly to mineral crystals. However, studies indicate that they act predominantly on cells involved in bone resorption, and not on mineral solubility. Thus, it has been proposed that BPs have cellular effects that cause osteoclast retraction, condensation, and cellular fragmentation (1 – 3) and induce apoptosis, which can be recognized by morphological changes in osteoclasts both in vitro (4 – 6) and in vivo (4). BPs also inhibit osteoclast recruitment and differentiation (7 – 12), the attachment of osteoclasts to the bone surface (13, 14), and ruffled border formation (15 – 17), which is essential for bone resorption. Notably, these effects may be a consequence of BPs interfering with the remodeling of the actin cytoskeleton (6, 18 – 20) and microtubule formation (18). However, BPs also affect osteoblasts, causing the release of a factor that inhibits osteoclast activity or formation (21 – 24). Although it is unclear which of these processes of bone resorption is most sensitive to BPs, it has been suggested that the most likely route by which BPs inhibit bone resorption is through a direct effect on resorbing osteoclasts (25). To clarify at which stages of osteoclast differentiation and activation that BPs influence, we have examined the osteoclasts generated from mononuclear precursors and osteoclasts in the calvaria by using a laser scanning confocal microscopy.

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Inhibition of osteoclast formation in bone marrow cell culture

Although there have been many investigations on the effect of BPs on the osteoclast differentiation and formation, the results remain controversial. For example, BP effects on resorption have been attributed to differences in osteoclastic activity rather than their number, based on apparently normal osteoclast formation in isolated rat osteoclast cultures (26). In these studies, alendronate suppressed the ability of osteo-

blastic cells to induce resorptive activity in osteoclasts and the inhibitory effect did not depend on resorptive release of alendronate from bone surfaces (26). Other studies have reported that the number and activity of osteoclast-like cells grown from murine bone marrow and in the tibia of BP-treated mice are reduced as a result of apoptosis (4). As observed previously (27, 28), reduced pseudopod formation is suggestive of an impaired ability of pre-osteoclasts to fuse, an important step in osteoclastic differentiation. To determine whether these responses were the result of direct effects of the BP on the osteoclasts, pre-osteoclasts derived from mouse bone marrow cells were incubated for 3 days in the absence or presence of 25 μM clodronate or 2.5 μM risedronate and stained with Alexa Fluor 488-conjugated phalloidin after fixation. Compared to the control (Fig. 1A), osteoclasts treated with BPs were much smaller and displayed retracted pseudopods (dotted arrows in Fig. 1, B and C) and were highly vacuolated (arrowheads in Fig. 1C). The small size of the BP-treated cells appeared to result from reduced cell fusion since the average number of nuclei in the tartrate resistant acid phosphatase (TRACP)-stained osteoclasts in the BP group was significantly lower than in control osteoclasts. The lower frequency of fusion of precursor cells in the BP group was also confirmed by measuring the area of osteoclasts that possess more than 3 nuclei per cell (Fig. 1D). Furthermore, both types of BP strongly inhibited the fusion of PKH 67 (green fluorescent dye)- or PKH26 (red fluorescent dye)-labeled living pre-osteoclasts (K. Suzuki et al., unpublished data). Thus, our studies of osteoclastogenesis using TRACP-positive mononuclear cells derived from bone marrow, which have been passed through Sephadex G-10 to remove stromal cells, has clearly shown that BPs inhibit macrophage-colony stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANKL)-induced fusion of osteoclast precursors in the absence of osteoblasts. Although the BPs may still inhibit the resorption-stimulating activity by osteoblasts, it is evident that BPs can act directly on osteoclast precursors to inhibit osteoclast differentiation.

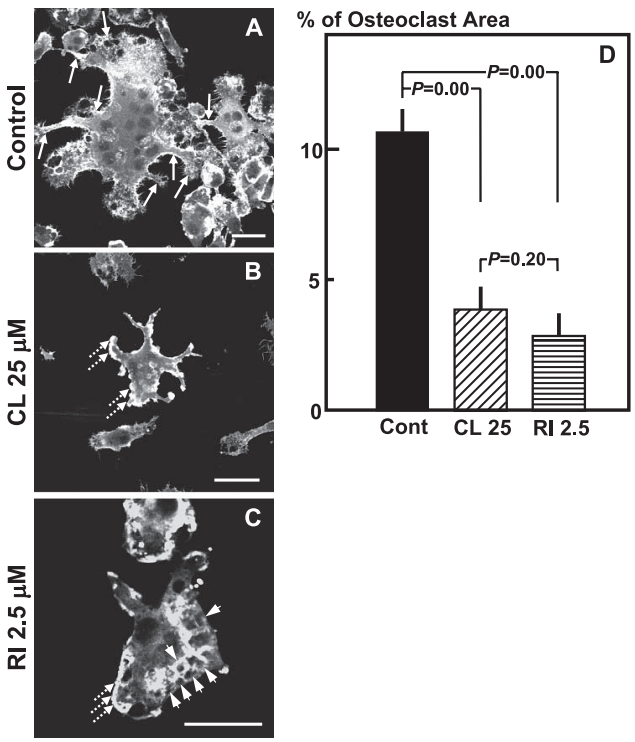

Fig. 1. Effects of BPs on the osteoclastogenesis. A – C: Pre-osteoclasts derived from mouse bone marrow cells were incubated for 3 days in the absence (A) or presence of 25 μM clodronate (B) or 2.5 μM risedronate (C) and stained for actin. Arrows, dotted arrows, and arrowheads indicate pseudopods extending from the osteoclasts, retracted pseudopods, and cytoplasmic vacuolization, respectively. Bars: 30 μm. Reproduced, with permission, from Ref. 34. D: Area measurement of TRACP-positive multinucleated (>3 nuclei) osteoclasts generated from mouse bone marrow cells in the absence (Cont) or presence of 25 μM clodronate (CL 25) or 2.5 μM risedronate (RI 2.5). Values are expressed as a percentage of the total culture area (mean ± S.D.; n = 8 – 11).

Mechanism of BP effects on bone resorption

The molecular mechanisms by which BPs inhibit osteoclast-mediated bone resorption have only recently been identified by Rogers et al.; their data suggest that simple BPs can be incorporated into non-hydrolysable analogues of ATP, whereas nitrogen-containing BPs inhibit farnesyl disphosphate synthase that is essential for the post-translational prenylation of small GTPase signaling proteins (25, 29, 30). Consistent with these observations, our immunocytochemical analyses of osteoclast cultures show that 0.1 – 2.5 μM risedronate impairs the perimembranous distribution of Rac1 and Cdc42, members of the Rho family GTPases, which are essential for the cytoskeletal rearrangement in osteoclast migration and fusion (31). Also our experiments using cholera toxin subunit B have revealed that intracellular form of osteopontin transiently colocalizes both with actin and membrane lipid rafts in fusing osteoclasts, indicating that osteopontin may act as a member of the signaling molecule complex, together with Rho family GTPases, in osteoclast fusion and differentiation. In addition to mediating cell adhesion, cell attachment molecules including osteopontin are known to signal through receptors and affect cytoskeletal remodelling and gene transcription. Thus, stimulation of the $\alpha v\beta 3$ integrin activates Shc2, Src family members (32) and other protein tyrosine kinases that mediate the intracellular signals. Moreover, we have shown that

osteopontin colocalizes with β3 integrin exclusively at the cell periphery and in the cell processes of fusing osteoclasts (31) and the large amount of osteopontin deposited onto the mineral in pre-osteoclast cultures seeded on the Osteologic™ coverslip was markedly reduced by BPs (unpublished data). Collectively, it is apparent that one of the inhibitory effects of BPs on bone resorption might be the impairment of the production of osteopontin and its localization at the cell periphery.

Changes in morphological features of calvarial osteoclasts induced by BPs

We have previously reported that monolayer cultures of pre-fusion osteoclasts extend many pseudopods and fuse into large multinuclear osteoclasts and that these processes are suppressed in the absence of osteopontin expression (28). To relate these observations to the behavior of osteoclasts in bone in situ, laser scanning confocal microscopy was used to examine neonatal mouse calvaria stained for TRACP activity after fixation to identify osteoclasts. Multinucleated, non-resorbing osteoclasts could be identified extending numerous long pseudopods used for generation of larger, multinucleated osteoclasts (arrows in Fig. 2A), as observed in monolayer cultures of osteoclasts (28). To study whether BPs affect the formation and differentiation of osteoclasts in bone, TRACP-stained calvaria were examined by confocal microscopy after culturing in the presence or absence of BPs. A wide range of morphological abnormalities were observed in the osteoclasts, ranging from mild to severe, within individual calvaria, even at the same concentration of BP. However, the frequency of the osteoclast abnormalities, including retraction of pseudopods and vacuolization of cytoplasm (arrowheads in Fig. 2B), was increased with increasing concentrations of BPs. Although cytoplasmic vacuolarization could reflect toxic effects of BPs, whether it is associated with apoptosis or causes the suppression of bone resorption activity is not clear at present.

To examine actively resorbing osteoclasts, calvaria were cultured for 48 h in the presence of 10^{-8} M parathyroid hormone (PTH) to stimulate resorption. Serial optical sections showed strong TRACP staining at the sealing zone (arrows in Fig. 2C) coinciding with the edge of resorption lacunae. Notably a similar morphology was also observed for bone-resorbing osteoclasts in calvaria treated with 10 μg/ml lipopolysaccharide (LPS) or 10^{-6} M prostaglandin E_2 (33). In calvaria cultured in the presence of LPS and 2.5 μM risedronate, the multinucleated osteoclasts were smaller, displayed severe cytoplasmic vacuolization (arrowheads in Fig. 2D), and the sealing zone was diffuse compared to osteoclasts in calvaria cultured with PTH alone (Fig. 2C). Furthermore, a reduction in the size and depth of resorption lacunae was evident from the quantitative analysis of the size of individual resorption bays and the % resorption (34).

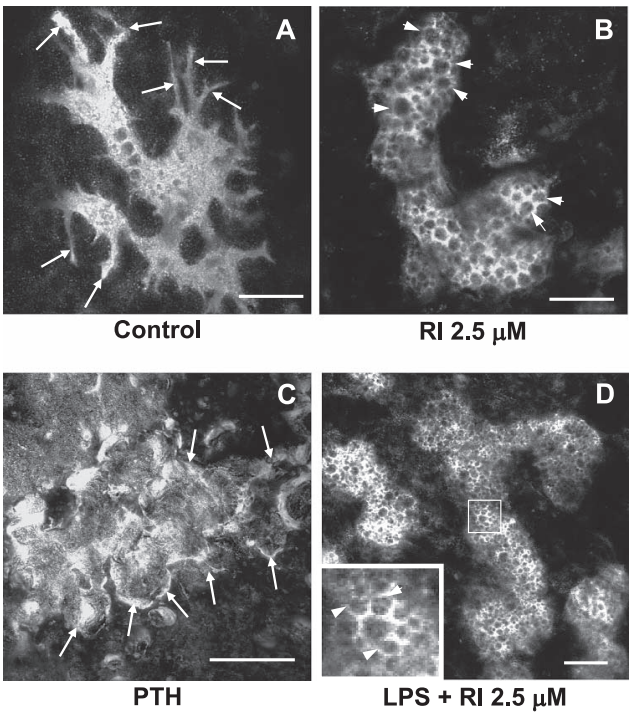

Fig. 2. Effects of BPs on the differentiation and activation of osteoclasts. Laser scanning confocal microscopy of neonatal mouse calvaria stained for TRACP activity after culturing in the presence of vehicle alone (A), 2.5 μM risedronate alone (B), 10^{-8} M PTH (C), or 10 μg/ml LPS and 2.5 μM risedronate (D). Arrows in panel A, arrows in panel C, and arrowheads indicate pseudopods extending from the osteoclasts, the edge of attachment zone, and cytoplasmic vacuolization, respectively. Bars: 30 μm (A, B), 200 μm (C), and 50 μm (D). Reproduced, with permission, from Ref. 34.

Effects of BPs on the colocalization of F-actin and β3 integrin/osteopontin in resorbing-osteoclasts

That cell adhesion molecules play an important role in skeletal growth, development, and homeostasis is well established. Cell attachment molecules, such as osteopontin and its $\alpha v\beta 3$ integrin receptor, are involved in osteoclast differentiation, migration to sites of resorption, fusion of post-mitotic osteoclast precursors, cellular polarization, and tight sealing zone formation required for bone resorption (35 – 40). Osteopontin binding to the $\alpha v\beta 3$ receptor induces rapid production of phosphatidylinositol triphosphate through phosphatidylinositol-3-kinase activation in association with c-src, which facilitates actin filament formation, osteoclast

motility, and bone resorption (41 – 43). Src-dependent tyrosine phosphorylation of Pyk2 is involved in the adhesion-induced formation of the sealing zone, required for osteoclastic bone resorption (44), resulting in the recruitment of cytoskeletal molecules such as paxillin, vinculin, gelsolin, and F-actin to adhesion contacts upon integrin activation (39). Thus, it is evident that osteopontin signaling through the $\alpha v\beta 3$ integrin directs cytoskeletal organization required for bone resorption. Although $\alpha v\beta 3$ integrin and osteopontin are known to play an important role in the attachment of osteoclasts to bone, we have been unable to show the expression of either protein associated with the actin ring in mature osteoclasts generated from bone marrow or spleen monocytes cultured on glass in the presence of macrophage-colony stimulating factor (M-CSF) and receptor activator of nuclear factor κB ligand (RANKL) (unpublished data). To investigate whether the difference in substrata to which the osteoclasts attach influences the expression of $\beta 3$ integrin and osteopontin, we examined resorbing-osteoclasts in the calvaria after double-staining for actin with $\beta 3$ integrin or osteopontin, by confocal microscopy. The actin and $\beta 3$ integrin show extensive co-localization and are concentrated in the actin ring bordering large resorption lacunae (arrowheads in Fig. 3A) in resorbing osteoclasts of calvaria

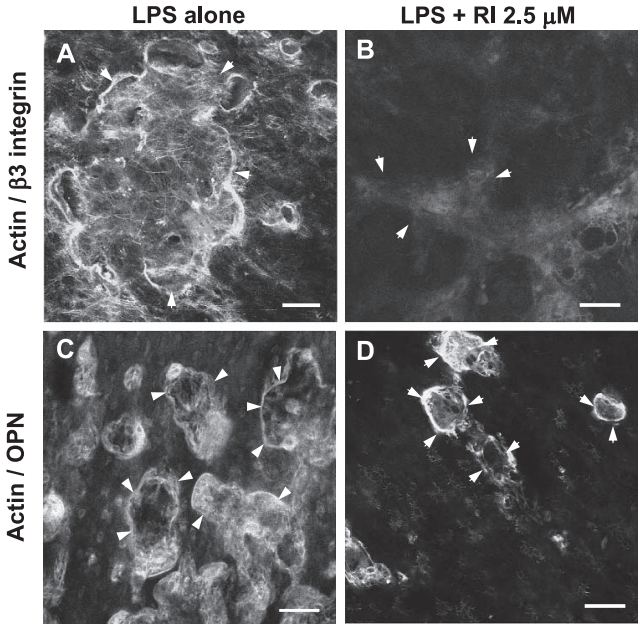

Fig. 3. Effects of bisphosphonates on the colocalization of actin and $\beta 3$ integrin in sealing zone of resorbing-osteoclasts in calvaria cultured in the presence of LPS. Laser scanning confocal microscopy of neonatal mouse calvaria stained for actin and $\beta 3$ integrin (A, B) or actin and osteopontin (C, D) after culturing for 48 h in the presence of 10 μg/ml LPS alone (A, C) or in combination with 2.5 μM risedronate (B, D). Arrowheads indicate the edge of resorption lacunae. Bars: 50 μm. Reproduced, with permission, from Ref. 34.

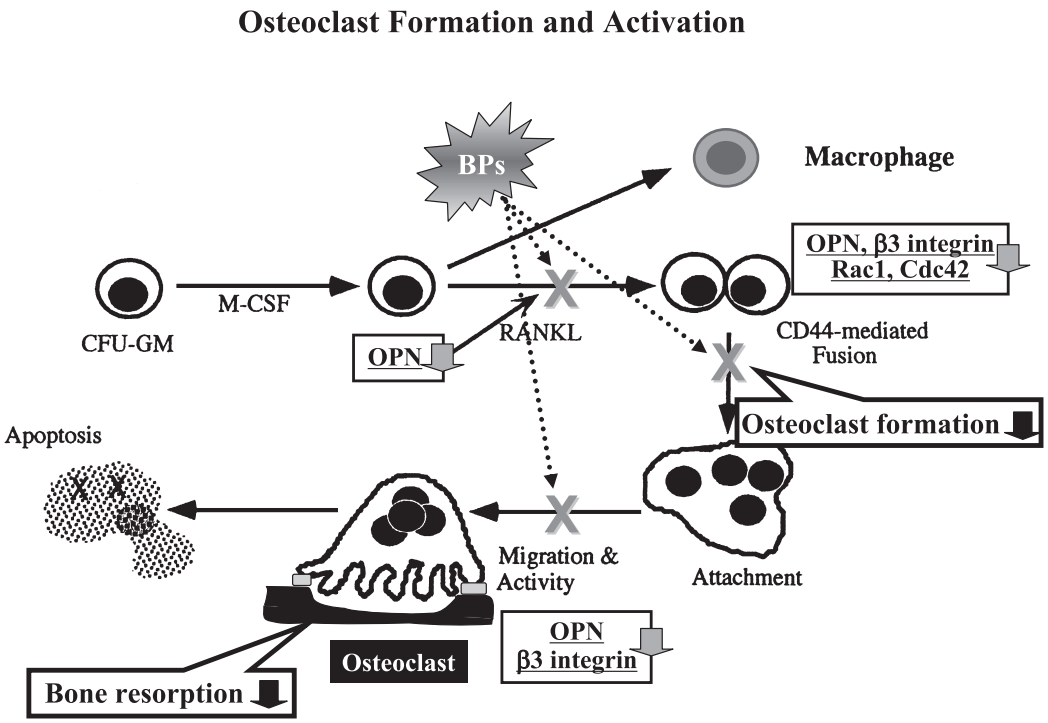

Fig. 4. A scheme for osteoclast formation and activation. Bisphosphonates inhibit the fusion, formation and activation of osteoclasts (dotted arrows and "X"), by impairing the distribution of osteopontin (OPN), $\beta 3$ integrin, Rac1, and Cdc42 at the periphery of fusing osteoclasts and osteopontin and $\beta 3$ integrin along the sealing zone of resorbing osteoclasts. CFU-GM, colony forming unit-granulocyte/macrophage.

cultured for 48 h in the presence of 10 μg/ml LPS. In BP-treated calvaria, the resorption lacunae were reduced in size and depth compared with the calvaria cultured with LPS alone (34). Furthermore, the presence of actin and β3 integrin along the edge of osteoclasts, which is required for cell attachment to the resorption site, was not readily observed (arrowheads in Fig. 3B).

Actin also co-localized with osteopontin (Fig. 3C), as observed in resorbing-osteoclasts stimulated by 10 μg/ml LPS. The co-localization was prominent at the periphery of the bone-resorbing osteoclasts and coincided with the edges of resorption lacunae (arrowheads in Fig. 3C), as seen with the actin and β3 integrin. In cultures treated with 2.5 μM risedronate, the resorption lacunae were reduced in size and depth (34), and the staining of actin and especially the osteopontin were markedly reduced and their peripheral co-localization lost (arrowheads in Fig. 3D). These results indicate that the cytoskeletal reorganization involved in the formation of a sealing zone around resorbing osteoclasts, which is strictly regulated by the substrata on which the cell locates, is one of the most susceptible processes in the inhibition by BPs.

Taken together, these findings indicate that BPs inhibit both formation and activation of osteoclasts, by impairing the distribution of osteopontin, β3 integrin, Rac1, and Cdc42, as depicted in Fig. 4.

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