Selective regulation of osteoclast adhesion and spreading by PLCγ/PKCα-PKCδ/RhoA-Rac1 signaling

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Bone resorption by multinucleated osteoclasts is a multistep process involving adhesion to the bone matrix, migration to resorption sites, and formation of sealing zones and ruffled borders. Macrophage colony-stimulating factor (M-CSF) and osteopontin (OPN) have been shown to be involved in the bone resorption process by respective activation of integrin αvβ3 via “inside-out” and “outside-in” signaling. In this study, we investigated the link between signal modulators known to M-CSF- and OPN-induced osteoclast adhesion and spreading. M-CSF- and OPN-induced osteoclast adhesion was achieved via activation of stepwise signals, including integrin αvβ3, PLCγ, PKCs, and Rac1. Osteoclast spreading induced by M-CSF and OPN was shown to be controlled via sequential activation, consistent with the osteoclast adhesion processes. In contrast to osteoclast adhesion, osteoclast spreading induced by M-CSF and OPN was blocked via activation of PLCγ/PKCα/RhoA signaling. The combined results indicate that osteoclast adhesion and spreading are selectively regulated via PLCγ/PKCα-PKCδ/RhoA-Rac1 signaling. [BMB Reports 2018; 51(5): 230-235]

INTRODUCTION

Osteoclast-mediated bone resorption is known to be a critical process in the development and physiology of the skeleton (1). Multinucleated mature osteoclasts repeatedly resorb old bone matrix and migrate to future bone resorption sites in a process referred to as the resorption cycle (2, 3). The resorption cycle of osteoclasts plays an important role in bone remodeling (4). In the initial stage of bone resorption, osteoclasts adhere to the bone surface via interactions with integrin, a large family of cell adhesion receptors. Integrin consists of α and β subunits and transmits cell-cell and cell-extracellular matrix (ECM) interactions (5). Binding of integrin to its ligand activates signal transduction pathways, which lead to cell adhesion, spreading, and cytoskeletal reorganization. Among integrin isoforms, integrin αvβ3 is predominantly expressed on the cytoplasmic surface of osteoclasts and interacts with bone matrix proteins such as osteopontin (OPN) and bone sialoprotein II (6) as well as participates in adhesion to bone, cytoskeletal reorganization, and bone resorption. It is also known that macrophage colony-stimulating factor (M-CSF) and hepatocyte growth factor (HGF) mediate osteoclast adhesion and spreading via integrin αvβ3-dependent mode (5). Coupling between αvβ3 and its binding partner triggers multiple signaling factors, such as phosphatidylinositol 3-kinase (PI3K), protein kinase C (PKC), phospholipase C (PLC), proline-rich tyrosine kinase (PYK2), c-Src, and small GTPases (7-12). Specifically, binding of M-CSF to its receptor, c-Fms, activates PLCγ and subsequent downstream signaling molecules, including PLCγ, PI3K, and small GTPases, via “inside-out” signaling (5, 7, 10). Further, direct interaction of OPN with αvβ3 was found to activate PYK2, c-Src, PI3K, and PLCγ via “outside in” signaling (10, 13) as well as enhance osteoclast survival and function by facilitating the Ca2+-dependent transcription factor NFATc1 pathway (14), which is essential for osteoclast differentiation.

PLCγ is a common downstream effector for integrin αvβ3- and M-CSF-mediated signaling in pre-fusion osteoclasts (10). Suppression of PLCγ2 has been shown to reduce cell adhesion, migration, and bone resorption in osteoclasts (15). PLC generates diacylglycerol and inositol-triphosphate, leading to PKC activation and calcium release from the endoplasmic reticulum (16). PKCα is thought to play a key role in integrin αvβ3-mediated signal transduction, osteoclast migration, and bone resorption (12). On the contrary, the functional roles of other PKC isoforms in osteoclast adhesion and spreading have not been fully understood. PKC is known to activate downstream small GTPase cascades involved in the modulation of integrin-mediated cytoskeletal organization (17, 18). For example, small GTPases (Rho and Rac) have been reported to organize and maintain cellular cytoskeletal...
structures in osteoclasts (19). Despite osteoclast adhesion and spreading being associated with various signal molecules, sequential coordination between signal modulators in osteoclast adhesion and spreading induced by M-CSF and OPN is not well understood. Here, we observed that consecutive activation of the PLCγ/PKCα3/Rac1 signal axis governs osteoclast adhesion and spreading induced by M-CSF and OPN. Further, the results show that signal activation via PLCγ/PKCα/RhoA plays a negative role in osteoclast spreading but not osteoclast adhesion.

RESULTS AND DISCUSSION

Osteoclast adhesion and spreading mediated by M-CSF and OPN share integrin αvβ3/PLCγ signaling

Growth factors and extracellular matrix proteins have been identified as activators for inducing cell adhesion and spreading in an integrin-dependent manner (5, 6, 10, 20). We first examined the effects of M-CSF and OPN on osteoclast adhesion and spreading. M-CSF and OPN promoted adhesion and spreading of mature osteoclasts (Fig. 1). M-CSF and OPN in osteoclasts are known to converge at integrin αvβ3 activation via “inside-out” signaling and “outside-in” signaling, respectively (5, 6). Thus, we investigated whether or not enhanced adhesion and spreading induced by M-CSF or OPN in mature osteoclasts are dependent upon integrin αvβ3. Treatment with functional blocking antibodies against integrins αv and β3 suppressed osteoclast adhesion and spreading in response to M-CSF and OPN (Fig. 2A and B). These results indicate that M-CSF and OPN facilitate osteoclast adhesion and spreading via an integrin αvβ3-dependent pathway. Since integrin αvβ3 was reported to activate PLCγ1 and PLCγ2 as well as foster their recruitment to αvβ3 in pre-fusion osteoclasts (10), we also examined whether or not PLCγ activation is required for M-CSF- and OPN-induced adhesion and spreading in osteoclasts. Treatment with PLCγ inhibitor (U73122) blocked M-CSF- and OPN-induced osteoclast adhesion and spreading in a dose-dependent manner (Fig. 2C and D). These results indicate that M-CSF- and OPN-induced osteoclast adhesion and spreading progress via “inside-out” signaling and “outside-in” signaling depending on integrin αvβ3, respectively, and share αvβ3 and PLCγ signaling.

PLCγ activates PKCα and PKCδ during osteoclast adhesion and spreading

PKCs are known to be downstream effectors of integrin-
mediated PLCγ signaling (21) and M-CSF has been reported to specifically stimulate PKCα and PKCβ among PKC isoforms in osteoclast precursors (22). Here, we investigated the relationship between PLCγ and PKC upon treatment with M-CSF or OPN in mature osteoclasts. PKCα and PKCβ were efficiently translocated into the osteoclastic cytoplasmic membrane in response to M-CSF or OPN (Fig. 3A and B). Moreover, phosphorylation levels of PKCα and PKCβ in membrane fractions were elevated after treatment with M-CSF or OPN. However, active forms of PKCα and PKCβ induced by M-CSF or OPN disappeared after treatment with PLCγ inhibitor (U73122). These findings indicate that M-CSF or OPN induces serial activation of PLCγ and PKCα/PKCβ signal during osteoclast adhesion and spreading. Further, we observed that osteoclast adhesion and spreading induced by M-CSF or OPN were suppressed by treatment with PKCα inhibitor (rottlerin) but not PKCβ inhibitor (Gö6976) (Fig. 3C and D). Interestingly, PKCα inactivation led to an approximately 1.5-fold increase in M-CSF- and OPN-induced osteoclast spreading compared to the control, indicating that PKCα negatively regulates osteoclast spreading. These results indicate that PKCβ is necessary for both osteoclast adhesion and spreading, whereas PKCα plays a negative role in osteoclast spreading.

**PKCα and PKCβ selectively regulate activities of RhoA and Rac1 during osteoclast adhesion and spreading**

Small GTPases have been reported to regulate integrin-dependent cell morphological changes, including formation of stress fibers, lamellipodia, and filopodia (5, 23, 24). It is also known that small GTPases (Rho and Rac) regulate cell spreading and cytoskeleton organization (2, 19). Since small GTPases are reported to be downstream effectors of the PKC signaling pathway (22, 25), we investigated whether or not M-CSF or OPN is involved in regulating the activities of small GTPases (RhoA and Rac1) in osteoclasts. As shown in Fig. 4A and B, elevation of RhoA activity by M-CSF or OPN was reduced by treatment with either PKCα inhibitor (Gö6976) or PKCβ inhibitor (rottlerin; 5 μM) (Fig. 4C and D). The RhoA inhibitor (Y27632; 10 μM) and Rac1 inhibitor (NSC23766; 100 μM) suppressed M-CSF- and OPN-mediated osteoclast adhesion and spreading (Fig. 4E). The effects of Rac1 inhibitor (Y27632; 10 μM) and Rac1 inhibitor (NSC23766; 100 μM) on M-CSF- and OPN-mediated osteoclast adhesion and spreading were assessed by the same procedures as described in the legend of Fig. 2. Data are expressed as the mean ± S.D. (n = 3) and presented as mean percentage relative to control. *P < 0.01; **P < 0.05. (E) Proposed model for M-CSF- and OPN-induced osteoclast adhesion and spreading. Integrin αvβ3 activation in response to M-CSF or OPN induces osteoclast adhesion via PLCγ/PKCα/Rac1 signal transduction. After cell adhesion, osteoclast spreading was activated via integrin αvβ3-mediated PLCγ/PKCα/Rac1 signaling but suppressed via PLCγ/PKCα/RhoA signaling.
PKCδ inhibitor (rottlerin). Specifically, the stimulatory effect of M-CSF and OPN on Rac1 activity was suppressed by treatment with PKCδ inhibitor but not PKCα inhibitor. These results indicate that PKCδ controls both RhoA and Rac1 activities, and PKCα regulates only RhoA activity during M-CSF- and OPN-induced osteoclast stimulation. Further, we observed that osteoclast adhesion and spreading induced by M-CSF or OPN were suppressed by treatment with Rac1 inhibitor (NSC23766) but not RhoA inhibitor (Y27632) (Fig. 4C and D). Particularly, RhoA inactivation led to an approximately 1.5-fold increase in M-CSF- and OPN-induced osteoclast spreading compared to the control, indicating that RhoA signaling is involved in inhibition of osteoclast spreading. The combined results indicate that PKCδ-mediated Rac1 activation is involved in both osteoclast adhesion and spreading, whereas PKCα-mediated RhoA activation negatively regulates osteoclast spreading but not osteoclast adhesion. Seesaw-like crosstalk between extracellular signal-regulated kinase (ERK) and p38 activation has been reported to occur during RANKL-induced osteoclastogenesis (26, 27). Treatment with p38 inhibitors resulted in increased ERK activation, and ERK inhibitors caused an increase in p38 activation. Consistent with these results, it is also possible that the treatment of PKCα or RhoA inhibitors may lead to the activation of PKCδ or Rac1.

For bone resorption, multinucleated osteoclasts derived from hematopoietic stem cells adhere to the bone surface via integrin ανβ3 present in the cytoplasmic membrane, resulting in the formation of a large and dense F-actin ring called the sealing zone and ruffled border (1, 2, 28). Osteoclast adhesion and subsequent spreading are critical in the initial stage of bone resorption by osteoclasts. Afterward, osteoclasts can resorb old or damaged bone by sequential repeated processes, including cell contraction, cell spreading by disassembling the sealing zone, and cell migration.

Here, we analyzed the regulatory mechanisms underlying osteoclast adhesion and spreading induced by M-CSF or OPN. As summarized in Fig. 4E, integrin ανβ3 in the surface of osteoclasts is activated by binding of M-CSF to c-Fms receptor in a process termed “inside-out” signaling. Further, OPN directly binds to and stimulates integrin ανβ3 in a process termed “outside-in” signaling. Integrin ανβ3 activated by M-CSF or OPN allows the initial adhesion of osteoclasts for bone resorption and subsequent spreading process via PLC/PKCα/Rac1 signaling. Additionally, we observed that PLC/PKCα/RhoA signaling stimulated by M-CSF or OPN plays a negative role in osteoclast spreading with no effect on osteoclast adhesion. Overall, our findings suggest that osteoclast adhesion and spreading are differentially regulated via PLC/PKCα/PKCδ/RhoA-Rac1 signaling.

MATERIALS AND METHODS

Antibodies and inhibitors
Antibodies specific for phosphorylated-PKCα and phosphorylated-
PKCδ were from Cell Signaling (Beverly, MA, USA); antibodies for PKCoα, PKCδ, integrins αν and β3, and actin were from Sigma-Aldrich (St. Louis, MO, USA). G06976, rottlerin, Y27632, and NSC23766 were from Calbiochem (San Diego, CA, USA).

Osteoclast differentiation
Bone marrow-derived monocytes were isolated from the long bones of 6-week-old C57BL/6 male mice by flushing the bone marrow cavity with minimum essential medium-alpha (α-MEM; HyClone, Logan, Utah, USA). Cells were centrifuged and the red blood cells were removed using red blood cell lysis buffer (Sigma-Aldrich). Next, cells were incubated with α-MEM containing 10% fetal bovine serum (FBS; HyClone) and M-CSF (5 ng/ml) for 12 h. The non-adherent cells were collected, plated on 100-mm culture dishes, and further cultured for 3 days with α-MEM containing M-CSF (30 ng/ml) to generate bone marrow-derived osteoclast precursors. Osteoclast precursors were differentiated into osteoclasts in α-MEM supplemented with M-CSF (30 ng/ml) and receptor activator of nuclear factor κB ligand (RANKL; 100 ng/ml) for 4 days with a change of medium on day 2.

Cell adhesion and spreading assays
Cell adhesion and spreading assays were performed as previously described (29). For M-CSF-induced osteoclast adhesion and spreading assays, osteoclasts were detached with cell dissociation solution (Sigma-Aldrich) and resuspended in α-MEM supplemented with M-CSF (30 ng/ml) and receptor activator of nuclear factor κB ligand (RANKL; 100 ng/ml) for 4 days with a change of medium on day 2. The extent of cell spreading was evaluated by measuring the surface area of osteoclasts using Image-Pro plus software.
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Small GTPases activation assays
Small GTPase activation assays were preformed using a small GTPase G-USA activation assay kit (Cytoskeleton Inc., Denver, CO, USA) according to the manufacturer’s procedures. To assess relative activities of small GTPases (RhoA and Rac1), the resultant absorbance was read at 450 nm using an ELISA microplate reader model 680 (Bio-Rad, Hercules, CA, USA).

Statistical analysis
All values are presented as means ± S.D. (n = 3). The difference among multiple groups was analyzed using one-way ANOVA analysis with Tukey’s test. P value of < 0.05 was considered to be significant. These analyses were performed using the program GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA).

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CONFLICTS OF INTEREST
The authors have no conflicting interests.

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