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The inhibitors of cyclin-dependent kinases and GSK-3β enhance osteoclastogenesis

Yosuke Akiba [a,1], Akiko Mizuta [b,1], Yoshito Kakihara [c,*], Juri Nakata [c,d], Jun Nihara [d], Isao Saito [d], Hiroshi Egusa [e], Makio Saeki [c]

[a] Division of Bio-Prosthodontics, Niigata University Graduate School of Medical and Dental Sciences, 2-5274 Gakkocho-dori, Chuoku, Niigata 951-8514, Japan
[b] Department of Pharmacology, Osaka University Graduate School of Dentistry, 1-8 Yamadaoka, Suita, Osaka 565-0871, Japan
[c] Division of Dental Pharmacology, Niigata University Graduate School of Medical and Dental Sciences, 2-5274 Gakkochodori, Chuoku, Niigata 951-8514, Japan
[d] Division of Orthodontics, Niigata University Graduate School of Medical and Dental Sciences, 2-5274 Gakkocho-dori, Chuoku, Niigata 951-8514, Japan
[e] Division of Molecular and Regenerative Prosthodontics, Tohoku University Graduate School of Dentistry, 4-1 Seiryo-machi, Aoba-ku, Sendai 980-8575, Japan

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ABSTRACT

Osteoclasts are multinucleated cells with bone resorption activity that is crucial for bone remodeling. RANK-RANKL (receptor activator of nuclear factor κB ligand) signaling has been shown as a main signal pathway for osteoclast differentiation. However, the molecular mechanism and the factors regulating osteoclastogenesis remain to be fully understood. In this study, we performed a chemical genetic screen, and identified a Cdks/GSK-3β (cyclin-dependent kinases/glycogen synthase kinase 3β) inhibitor, kenpaullone, and two Cdks inhibitors, olomoucine and roscovitine, all of which significantly enhance osteoclastogenesis of RAW264.7 cells by upregulating NFATc1 (nuclear factor of activated T cells, cytoplasmic 1) levels. We also determined that the all three compounds increase the number of osteoclast differentiated from murine bone marrow cells. Furthermore, the three inhibitors, especially kenpaullone, promoted maturation of cathepsin K, suggesting that the resorption activity of the resultant osteoclasts is also activated. Our findings indicate that inhibition of GSK-3β and/or Cdks enhance osteoclastogenesis by modulating the RANK–RANKL signaling pathway.

1. Introduction

Bone homeostasis is tightly controlled by osteoblasts and osteoclasts which are involved in bone formation and resorption, respectively [1–3]. The imbalance between bone formation and resorption leads to impaired bone remodeling and development of bone disorders. Enhanced bone resorption by osteoclasts weakens bone structure and can cause osteoporosis over time whereas defects in the function of osteoclasts lead to osteopetrosis [1]. Osteoclasts are giant multinucleated cells derived from monocyte/macrophage lineage precursor cells through the differentiation process primarily induced by two cytokines, macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor κB ligand (RANKL), which are produced by osteoblasts [4]. The M-CSF supports proliferation and survival of osteoclast precursor cells, and upregulates RANK expression. The RANKL and RANK interaction recruits adaptor protein TRAF6, which in turn assembles with TAB2-TAK1 to activate mitogen-activated kinases (MAPKs) such as extracellular signal-regulated kinase (ERK), Jun N-terminal kinase (JNK), and p38 as well as NF-κB pathways [5]. NF-κB is required for initial induction of NFATc1, a key transcription factor for osteoclast differentiation. Then, MAPKs activate AP-1 (c-Fos/c-Jun), that further amplifies NFATc1 [6]. The activity of NFATc1 is regulated by calcium signaling that is induced by activation of the immunoglobulin-like receptors associated with the immunoreceptor tyrosine-based activation motif (ITAM)-harboring adaptor proteins, including DAP12 and FcRγ [7]. The activated calcineurin dephosphorylates NFATc1, which subsequently translocates to nucleus and cooperatively induces osteoclast-related genes with other transcription factors such as MITF, PU.1, CREB, and AP-1 [8]. Thus, the RANK–RANKL signaling activates various downstream signaling pathways required for the osteoclastogenesis [9]. Recent studies have significantly advanced our knowledge about the regulatory mechanism of osteoclastogenesis pathway, but the whole osteoclastic signaling network is yet unknown. Therefore, we performed a chemical genetic screen to identify novel pathways and factors which controls osteoclastogenesis by using annotated small compounds LOPAC^{1280} (Sigma). Our screen

* Corresponding author.
E-mail address: kakihara@dent.niigata-u.ac.jp (Y. Kakihara).
[1] These authors equally contributed to this work.

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identified a Cdk/GSK-3β inhibitor, kenpaullone, and two Cdk inhibitors, olomoucine and roscovitine, as activators of the osteoclastogenesis. Our data showed that the inhibition of Cdks and/or GSK3-β significantly upregulates NFATc1 and subsequently enhances the formation of functional osteoclasts.

2. Material and methods

2.1. Cells and reagents

For the osteoclastogenic culture, RAW264.7 and pNFAT/Luc-RAW cells were cultured in α-MEM medium containing 10% fetal bovine serum (FBS), 100 ng/ml soluble RANKL (sRANKL, Peprotech and Oriental Yeast), 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin [10]. For osteoclast formation by using primary pre-osteoclast cultures, murine bone marrow cells were obtained from femurs and tibiae of 7-week-old ddY mice (Japan SLC, Inc.). 4.0×10^5 cells were cultured in α-MEM medium containing 10% fetal bovine serum (FBS), 100 ng/ml sRANKL, 10 ng/ml M-CSF (Wako, JPN), 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin in 24-well plates. Kenpaullone (Sigma), olomoucine (Sigma), roscovitine (Sigma) are used at a final concentration of 50 nM. The culture medium in each well was replaced by fresh medium containing M-CSF and sRANKL every 2 days. TRAP staining was performed after 5 days of the induction.

2.2. Cell-based screening of the small-compound library

The pNFAT/Luc-RAW cells were used for screen small compounds, LOPAC^{1280} (Sigma) that contains 1280 compounds of marketed drugs and pharmaceutically relevant structural derivatives. These compounds are annotated with biological activities and classified as follows: cell signaling (9%), phosphorylation (8%), cell stress (4%), lipids (4%), ion channels (6%), G proteins (3%), apoptosis/cell cycle (2%), gene regulation (3%), hormone related (3%), and neuroscience related (58%). The pNFAT/Luc-RAW cells (5000 cells/well) were plated into 96-well plates in 100 μl of α-MEM medium with 10% FBS, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. The culture medium was exchanged the next day for fresh medium containing sRANKL (100 ng/ml), followed by the addition of each compound in the library to the cells at 10 μM. The luciferase activity of each well was measured after 24 h using the ONEGlo™ luciferase assay system (Promega) and a microplate reader (GloMax-Multi Detection System, Promega).

2.3. TRAP staining

TRAP staining was performed as described previously [11]. Briefly, the cells were fixed with 10% glutaraldehyde for 15 min at 37 °C, and subsequently incubated for 10 min at 37 °C in TRAP buffer, which consisted of 0.1 M sodium acetate, 0.1 M acetic acid, 10 mg/ml naphthol AS-MX phosphate (Sigma), 0.1% Triton X-100 (Sigma), 0.3 M potassium tartrate (Sigma) and 0.3 mg/ml Fast Red Violet LB Salt (Sigma). TRAP-positive dark-red cells with more than three nuclei were counted under light microscope as multinucleated osteoclasts.

2.4. Quantitative real-time PCR

Total RNA was extracted with an RNeasy Mini Kit (QIAGEN, Hilden, Germany). After DNase I treatment (Ambion, Austin, TX), cDNAs were synthesized from 2 μg of total RNA using Super ScriptIII reverse transcriptase (Invitogen, Carlsbad, CA). Quantitative TaqMan® real-time PCR analysis for expression of NFATc1 was performed using the AB 7300 real-time PCR system (Applied Biosystems, Foster City, USA). The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as an internal control. The TaqMan® primer and probe sets used were Mm00479445_ml (NFATc1) and 4352339E (GAPDH).

2.5. Western blotting

The cells were harvested and lysed in Laemmli sample buffer [pH6.8; 50 mM Tris–HCl, 2% sodium dodecyl sulfate (SDS), 10% glycerol, 5% 2-mercaptoethanol] and heated at 95 °C for 5 min. Equal amounts of whole cell lysates were loaded and resolved via SDS-PAGE, and subjected to Western blotting analysis by using anti-NFATc1 monoclonal (7A6, Santa Cruz Biotechnology, Santa Cruz, CA), anti-cathepsin K polyclonal (ab19027, Abcam), and anti-β-actin monoclonal antibodies (A5441, Sigma). The signals were detected with an enhanced chemiluminescence (ECL) plus kit (GE Healthcare).

2.6. MTT assay

RAW264.7 cells were seeded onto 96-well plates at 50,000 cells/ml in 90 μl α-MEM complete medium. The cultures were incubated in the presence of 0.1% DMSO, 5 μM kenpaullone, 5 μM olomoucine, or 5 μM roscovitine for 0 (6 h), 1, 2, and 3 days followed by the addition of 10 μl of 5 mg/ml MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) (Sigma). After 4 h of incubation, 100 μl of SDS-HCl solution was added and incubated for 24 h to dissolve the formazan produced by the cells. Optical densities (OD) were measured at a wavelength of 571 nm.

3. Results

3.1. Screen of annotated small compounds modulating osteoclastogenesis of RAW264.7

Previously, we constructed RAW264.7 reporter cell line (pNFAT/Luc-RAW) that stably expresses luciferase under the control of NFAT-response element activation-dependent promoter [10]. By using this cell line, we screened the small compound Library of Pharmacologically Active Compounds (LOPAC^{1280}, Sigma) to identify drugs which modulate osteoclastogenic pathway. The cells were treated with sRANKL in the presence of 10 μM of each library compound or DMSO as a control, and the luciferase activity was measured after 24 h of incubation ($n=3$). Relative luciferase activity is defined as $\log_{10}$ ratio of the activity in the presence of compound vs. the solvent (DMSO). As shown in Fig. 1A, a number of compounds which significantly enhance or inhibit the NFAT-luciferase activity were identified. In this study, we focused our analysis on the activators for osteoclastogenesis. The top 30 activator candidates for osteoclastogenesis are listed in Table 1. Kenpaullone (9-bromo-7,12-dihydroindolo[3,2-d][1]benzazepin-6(5H)-one), a cyclin-dependent kinases (Cdks)/glycogen synthase kinase 3β (GSK-3β) inhibitor, showed the most significant activity in this screen. As shown in Fig. 1B, the kenpaullone enhanced NFAT-luciferase activity in a concentration-dependent manner in the range of which we tested (0–10 μM). Furthermore, to see the effect of kenpaullone on endogenous NFATc1 mRNA expression levels, we induced osteoclast formation in RAW264.7 cells with sRANKL in the presence (5 μM) or absence of kenpaullone for 4 days, and subsequently analyzed the NFATc1 mRNA levels by real-time RT-PCR. The mRNA levels were normalized by the levels of control sample (no sRANKL). As shown in Fig. 1C, the NFATc1 mRNA expression was significantly enhanced in the presence of kenpaullone compared to the cells without the compound.