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Extracellular acidosis accelerates bone resorption by enhancing osteoclast survival, adhesion, and migration

Heejin Ahn [1], Jin Man Kim [1], Kyunghee Lee, Hyunsoo Kim, Daewon Jeong *

Department of Microbiology, Aging-associated Vascular Disease Research Center, Yeungnam University College of Medicine, Daegu 705-717, Republic of Korea

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ABSTRACT

Acidic extracellular pH promotes osteoporotic bone loss by osteoclast activation. However, the change of osteoclastic cell behavior in acidosis-stimulated bone resorption process is unknown. We found that lowering extracellular pH induced an increase in the survival, adhesion, and migration of mature osteoclasts with a full actin ring, leading to enhanced pit formation on dentine slices. Acidosis upregulated osteopontin, which is an Arg-Gly-Asp (RGD) motif-containing matrix protein secreted from osteoclasts and acts as a common modulator for their survival, adhesion, and migration. A synthetic RGD peptide treatment blocked acidosis-induced osteoclast adhesion and migration, likely by competing with the RGD motif-containing extracellular matrix proteins for cell surface integrin binding. We finally observed that acidosis was associated with activation of osteoclast survival/adhesion/migration-related Pyk2, Cbl-b, and Src signals. Collectively, the findings indicate that extracellular acidosis stimulates bone resorption by extending osteoclast survival and facilitating osteoclast adhesion and migration.

1. Introduction

Maintenance of physiological pH within and outside cells is critical for normal cell functioning. A shift in pH toward a more acidic environment under various conditions can lead to the following two consequences: (i) systemic acidosis, which is caused by a variety of pathological conditions such as renal and respiratory disease, diabetes, anemias, menopause, and aging, results in abnormal cell function in the whole body [1], and (ii) localized extracellular acidosis, which occurs under both physiological and pathological conditions, such as a ligand–receptor reaction arising in a microenvironment region, inflammation, infection, wound healing, and tumors [1–3]. Such acidosis seems to play a positive or negative role in infectious disease, early wound healing, tumorigenesis, and bone remodeling, but the mechanism is poorly characterized. Extracellular acidosis promotes an inflammatory cell defense process against pathogens via migration and phagocytosis [4]. In contrast, lactic acidosis due to increased glycolysis during tumorigenesis facilitates tumor invasion and metastasis [5,6], leading to deleterious impacts on biological processes.

Bone resorption by mature osteoclasts requires multiple sequential processes [7–9], including adhesion to the bone matrix, specialized sealing zone formation, local acidification of bone resorption lacuna, mineral dissolution and organic matrix degradation, and subsequent migration of osteoclasts to another resorption site. Acidosis has both harmful and beneficial effects on bone remodeling, depending on the systemic or localized contribution. Systemic acidosis, mainly caused by renal tubular acid excretion and pulmonary CO_2 exhalation defects, induces bone loss [1,10,11]. This observation is consistent with *in vitro* experiments showing that extracellular acidosis inhibits osteoblast-mediated biomineralization [12] and stimulates osteoclastic bone resorption [1,10], probably as a result of up-regulation or stabilization of osteoclastogenic and bone-resorptive factors, such as NFATc1 [13], carbonic anhydrase II [14], vacuolar-type H^+-ATPase [15,16], and osteopontin [17]. Systemic acidosis leads to osteoporotic bone defects, resulting from the opposing action of osteoblast inactivation and osteoclast activation. During normal bone resorption, osteoclasts display a specialized sealing zone through a tight interaction between bone surface matrix proteins and cell surface integrins, particularly $\alpha v\beta 3$, and form the lacuna, which is a bone-resorbing area [18,19]. Local acidosis in the resorption lacuna between the plasma membrane (ruffled border) and the bone surface occurs as a result of a vacuolar-type H^+-ATPase proton pump localized to the ruffled border membrane [15,20] and leads to dissolution of alkaline bone minerals and degradation of the organic bone matrix by acidic proteinases, including collagenase and cathepsin K secreted by osteoclasts [7,21].

Despite the stimulatory action of extracellular acidosis on osteoclast-mediated bone resorption, osteoclast behaviors regulated by acidosis are uncertain. Due to a technical problem forming

* Corresponding author. Address: Department of Microbiology, Yeungnam University College of Medicine, 317-1 Daemyung-Dong, Nam-Gu, Daegu 705-717, Republic of Korea. Fax: +82 53 653 6628.
E-mail address: dwjeong@ynu.ac.kr (D. Jeong).
[1] These authors contributed equally to this study.

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local acidosis similar to bone resorption lacuna, we used HEPES-buffered culture media with a lower pH-buffering capacity to evaluate the role of extracellular acidosis in the regulation of osteoclast behavior during the resorption phase.

2. Materials and methods

2.1. Medium preparation and cell culture

Cells were maintained under a humidified atmosphere of 5% CO_2 at 37 °C in bicarbonate-buffered α-MEM (Thermo Scientific, Rockford, IL, USA) supplemented with 10% fetal bovine serum (FBS) and antibiotics. To induce efficient extracellular acidosis, bicarbonate-free α-MEM (cat no. 11900-016; Invitrogen, Carlsbad, CA, USA) buffered with 10 mM HEPES and supplemented with 10% FBS and antibiotics was prepared by adjusting the pH to 7.0 or 7.5 with 1 M NaOH followed by filtering with a 0.22 pore size filter. Cells exposed to HEPES-buffered medium were grown in a humidified atmosphere without CO_2 at 37 °C.

2.2. Osteoclast differentiation, survival, and bone pit formation assay

Bone marrow-derived macrophages (BMMs) were obtained as osteoclast precursors using the following procedures. The long bones from 6-week-old C57BL6 male mice (Central Lab Animals, Korea) were flushed, incubated with bone marrow monocytes in α-MEM containing 5 ng/ml macrophage colony stimulating factor (M-CSF) for 12 h, and the adherent monocytes were cultured in α-MEM containing 30 ng/ml M-CSF for 3 days. Osteoclast precursors were further differentiated into multinuclear osteoclasts in 30 ng/ml M-CSF and 100 ng/ml receptor activator of nuclear factor-κB ligand (RANKL) for 4 days with a change of medium after 2 days. After the cells were stained with tartrate-resistant acid phosphatase (TRAP) using a leukocyte acid phosphatase staining kit (Sigma–Aldrich, St. Louis, MO, USA), the survival of osteoclasts was determined by counting TRAP-positive multinucleated cells [TRAP(+) MNCs] with more than 3 or 10 nuclei and a full actin ring. For bone resorption assay, osteoclast precursors were seeded on dentine slices (IDS Ltd., Boldon, UK) pre-wetted with culture medium and differentiated into mature osteoclasts for the pit formation assay. After changing the HEPES-buffered medium (pH 7.0 or 7.5) containing M-CSF and RANKL, osteoclasts were further cultured under a CO_2-free condition for the indicated times to allow bone resorption. Alternatively, osteoclasts were detached from the culture dish using a cell dissociation solution (Sigma–Aldrich), resuspended in HEPES-buffered medium containing M-CSF and RANKL, and seeded on dentine slices followed by a further incubation under a CO_2-free condition to resorb bone. After cells present on the surface of dentine slices were removed by ultrasonication, and the slices were stained with hematoxylin (Sigma–Aldrich), the area of resorbed pits was analyzed using Image-Pro Plus version 6.0 software (MediaCybernetics, Silver Spring, MD, USA).

2.3. Osteoclast adhesion and migration assay

Forty-eight-well culture plates were coated with or without vitronectin (20 μg/ml; BD Bioscience Sparks, MD, USA) for 2 h at 37 °C, washed with PBS, and then blocked with 1% bovine serum albumin (Invitrogen) in PBS for 1 h at 37 °C. Detached and purified osteoclasts were resuspended in HEPES-buffered medium (pH 7.0 or 7.5) in the presence of M-CSF and RANKL and seeded at a density of 2×10^5 cells per well in 48-well plates coated with or without vitronectin. Cells were incubated at 37 °C for 1 h, the non-adherent cells were removed by aspiration, washed with PBS, fixed with 3.7% formaldehyde, and stained with TRAP. Then, the extent of osteoclast adhesion was assessed by counting TRAP(+) MNCs under a light microscope. Cell migration was determined using 8 μm pore size and 24-well Transwell chambers (Corning Inc., NY, USA), as described previously [17], with modifications. Osteoclasts were resuspended in HEPES-buffered medium supplemented with M-CSF and RANKL and seeded at a density of 2×10^4 cells per well in a Transwell chamber coated or not with vitronectin (20 μg/ml). Cells were incubated under a CO_2-free condition for 6 h, fixed with 3.7% formaldehyde, and stained with TRAP to visualize migrated osteoclasts. Cells on the upper surface were removed using a cotton swab, and the migrated cells on the lower surface were counted.

2.4. Immunoblotting analysis

Osteoclasts formed in cultures with M-CSF and RANKL were resuspended in HEPES-buffered serum-free medium (pH 7.0 or 7.5) and cultured in 60 mm culture dishes for the indicated times. Cells were washed twice with ice-cold PBS and lysed in a lysis buffer containing 20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 1 mM EDTA, 1 mM NaF, 1 mM Na_3VO_4, 1 mM β-glycerol phosphate, and 1× protease inhibitor cocktail (Roche, Mannheim, Germany). Whole cell lysates were centrifuged, and the resulting supernatants were subjected to SDS–PAGE and immunoblotting with phospho-Src (Tyr 416) antibody (Cell Signaling Technology, Danvers, MA, USA). The resulting supernatants were precleared by incubating with Protein A/G PLUS-Agarose (Santa Cruz Biotechnology, CA, USA) for 1 h at 4 °C on a rocker to detect phospho-Pyk2 and phospho-Cbl-b. After centrifugation, precleared lysates were incubated with specific antibodies to Pyk2 and Cbl-b (Santa Cruz Biotechnology) overnight at 4 °C followed by further incubation with Protein A/G PLUS-Agarose beads for 2 h and centrifugation. The precipitates were subjected to SDS–PAGE and immunoblotting with 4G10 anti-phosphotyrosine antibody (Millipore, Bedford, MA, USA). The specific band was detected using appropriate HRP-conjugated second antibodies (Cell Signaling Technology) and enhanced chemiluminescence reagents (Ab Frontier, Seoul, Korea).

2.5. Statistical analysis

Data are presented as mean ± SD and compared with the two-tailed Student's *t*-test to analyze differences among groups. A *P*-value <0.05 was considered significant.

3. Results and discussion

3.1. Extracellular acidosis stimulates osteoclast bone pit formation

We prepared 10 mM HEPES-buffered media (pH 7.5 or 7.0) in place of $NaHCO_3$-buffered media to generate acidification by differences in extracellular pH within a physiological range. The HEPES-buffered system with a lower buffering capacity than that of a $NaHCO_3$-buffered system is advantageous to observe the effect of a pH change on cell behavior and function. In agreement with our previous reports [17], relatively low pH (pH 7.0) in the culture medium induced an increase in osteoclast formation compared to that in pH 7.5 medium (Supplementary Fig. 1), likely by stimulating adhesion and migration of osteoclast progenitors (BMMs) (Supplementary Fig. 2). Both cell adhesion and migration are a critical step in osteoclast formation and bone-resorbing processes of mature osteoclasts [7,8]. Osteoclasts adhere to the bone surface at the sealing zone and acidify the resorptive microenvironment (resorption lacuna) to induce mineral dissolution and matrix degradation, and then move towards another resorption site. Hence,