Biochemical and Biophysical Research Communications 418 (2012) 144–148

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

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

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ARTICLE INFO

Article history:
Received 27 December 2011
Available online 8 January 2012

Keywords:
Acidosis
Extracelluar matrix protein
Integrin
Osteoclast bone resorption

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

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doi:10.1016/j.bbrc.2011.12.149

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,

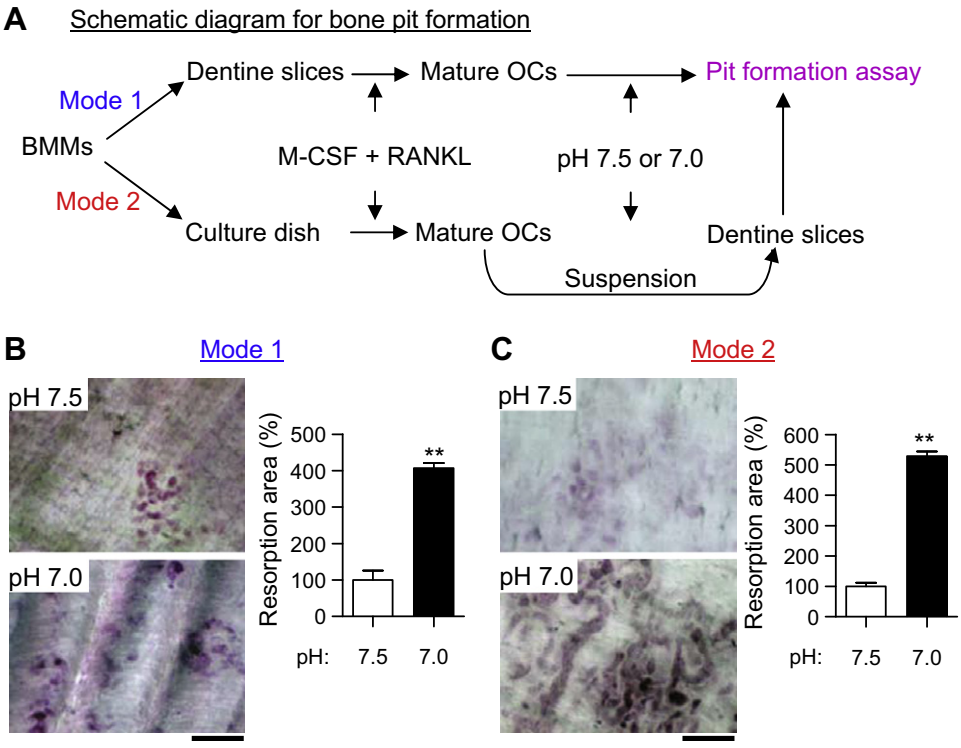

Fig. 1. Stimulatory effect of acidic extracellular pH on osteoclast-mediated bone pit formation. (A) Schematic diagram for bone pit formation. Mode 1: osteoclast precursors (BMMs) were differentiated into mature osteoclasts on dentine slices in 96-well plates and then replenished with HEPES-buffered medium (pH 7.0 or 7.5) containing M-CSF and RANKL. Mode 2: detached osteoclasts from the culture dish were resuspended in HEPES-buffered medium with M-CSF and RANKL and plated on dentine slices in 96-well culture plates. Osteoclasts exposed to HEPES-buffered medium were transferred to a CO_2-free atmosphere and incubated for 40 h to allow pit formation. (B) Pit formation assay via Mode 1. (C) Pit formation assay via Mode 2. Data are mean ± SD. $^{**}P < 0.01$ vs. pH 7.5. Scale bar = 50 μm.

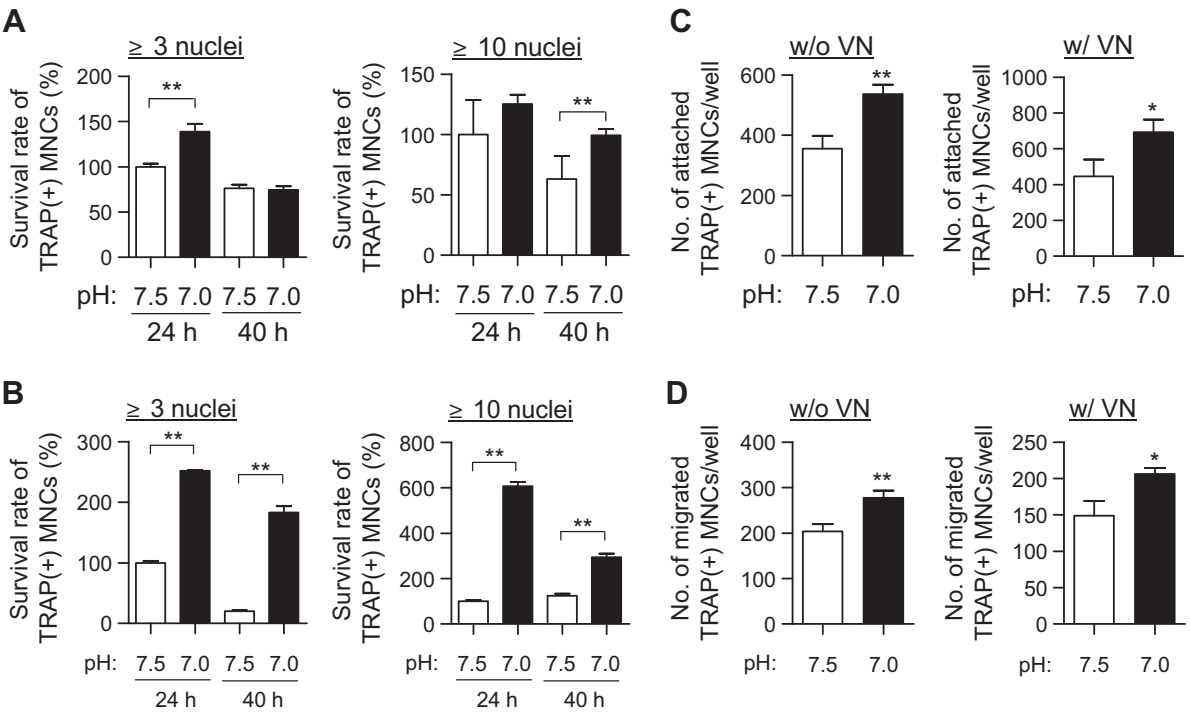

Fig. 2. Extracellular acidosis enhances osteoclast survival, adhesion, and migration. (A and B) Osteoclast survival assay. To assess osteoclast survival under conditions similar to the pit formation assay in Fig. 1A, the survival assay was executed using two different procedures. (i) Osteoclast precursors were differentiated into osteoclasts in 48-well culture plates and then directly exposed to HEPES-buffered medium with M-CSF and RANKL (A). (ii) Detached osteoclasts from the culture dish were resuspended in HEPES-buffered medium with M-CSF and RANKL and seeded on 48-well culture plates. After osteoclasts prepared from the two different methods were incubated under a CO_2-free condition for 24 or 40 h and stained with TRAP, osteoclast survival was determined by counting [TRAP(+) MNCs] with more than 3 or 10 nuclei and a distinct actin ring. Data as in (A) and (B) are mean ± SD. $^{**}P < 0.01$. (C) Osteoclast adhesion assay. (D) Osteoclast migration assay. Osteoclast adhesion and migration were examined using a 48-well culture plate and a 24-well Transwell chamber coated with (w/) or without (w/o) vitronectin (VN), respectively. Data as in (C) and (D) are mean ± SD. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. pH 7.5.

we first evaluated whether the pH change differentially regulated osteoclast bone-resorbing activity. When osteoclasts cultured on dentine slices were replenished with HEPES-buffered media and transferred to a CO_2-free condition (Fig. 1A: Mode 1), pit formation by osteoclasts increased at pH 7.0 compared to that at pH 7.5 (Fig. 1B). Because adherent osteoclasts on dentine slices received media of a different pH, this approach does not exclude the possibility that the pH change might have affected osteoclast adhesion. Thus, detached osteoclasts from a culture dish were resuspended in HEPES-buffered media and then applied to the pit formation assay (Fig. 1A: Mode 2). As a result, pit formation also increased at pH 7.0 compared to that at pH 7.5 (Fig. 1C). These results indicate that a pH shift in the extracellular environment towards a more acidic condition facilitates the osteoclastic bone-resorbing function.

3.2. Extracellular acidosis improves osteoclast survival, adhesion, and migration

As acidosis affects osteoclast formation by tuning cell adhesion and migration (Supplementary Figs. 1 and 2) and stimulates osteoclast function (Fig. 1), we next tested osteoclast survival and its cell behaviors, including adhesion, spreading, and migration, which are key players capable of determining bone-resorptive functions of osteoclasts. When osteoclasts formed by RANKL were exposed to HEPES-buffered media at pH 7.0 or 7.5, those containing more than 3 or 10 nuclei and an apparent actin ring lasted for a longer time at a relatively low pH (Fig. 2A). Additionally, when resuspended mature osteoclasts in HEPES-buffered media were exposed to a CO_2-free atmosphere to analyze osteoclast survival, osteoclasts had a longer lifetime in culture media at pH 7.0 than that at pH 7.5 (Fig. 2B). The latter experiment showed a higher survival efficacy of osteoclasts than the former, suggesting the possibility that an acidic environment may aid in environmental adaptation via cell adhesion and lead to extended osteoclast survival. In the present experiment, increased osteoclast lifetime in an acidic environment did not rule out newly formed osteoclasts during the bone resorption process, because acidic pH promotes osteoclast formation (Supplementary Fig. 1). Furthermore, when osteoclast adhesion and migration were determined in culture dishes and Transwell chambers coated or not with an N-terminal Arg-Gly-Asp (RGD) tripeptide motif-containing vitronectin, osteoclast adhesion and migration increased at pH 7.0 relative to that at pH 7.5 (Fig. 2C and D), but osteoclast spreading was not associated with pH (Supplementary Fig. 3).

3.3. Augmented osteoclast adhesion and migration by a relatively low pH depends on the interaction between extracellular matrix protein and its cognate receptor

The close coordination between integrin αvβ3 and RGD-containing bone matrix proteins including vitronectin and osteopontin tunes osteoclast function [18,19]. Our previous report showed that low pH medium induces increased osteopontin expression during RANKL-stimulated osteoclast differentiation [17]. We also found a similar pattern that pH 7.0 medium induced a marked increase in osteopontin secreted into the extracellular space of mature osteoclasts compared to that of medium at pH 7.5 (Fig. 3A). To further explore the role of bone matrix proteins such as osteopontin in osteoclast behavior, we used a synthetic RGD peptide to antagonize matrix protein function by competing with the αvβ3 receptor. Treatment with the RGD peptide prevented an increase in osteoclast adhesion and migration under decreased medium pH (Fig. 3B and C). In concert with the stimulatory action of acidic pH on osteoclast adhesion and migration, acidic pH induced prominent activation of the Pyk2, Cbl-b, and Src signals (Fig. 4), which are involved in osteoclast adhesion, migration, and survival

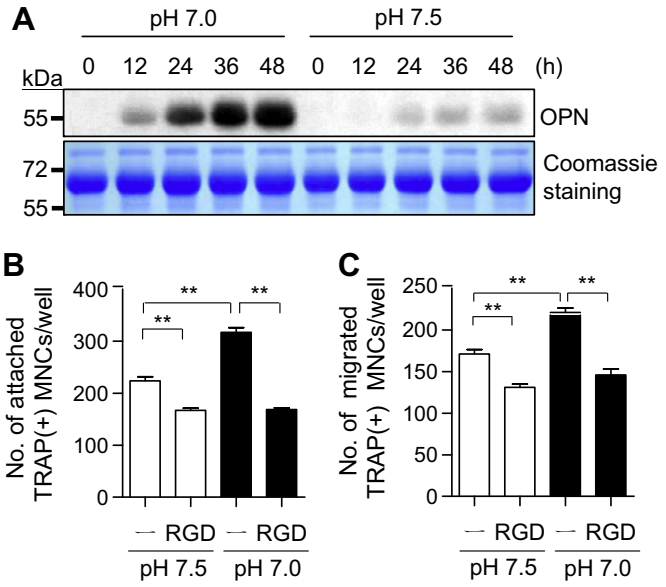

Fig. 3. Acidic pH upregulates the expression of osteopontin containing the Arg-Gly-Asp (RGD) motif, and osteoclast adhesion and migration is dependent on RGD binding. (A) The expression level of secreted osteopontin (OPN) in culture media. Osteoclasts were cultured under a CO_2-free condition in HEPES-buffered medium supplemented with 0.5% fetal bovine serum in the presence of M-CSF and RANKL for the indicated times. An equal volume of culture media was subjected to SDS–PAGE and immunoblotting with an OPN specific antibody. The Coomassie-stained band was used as a loading control. (B) Inhibition of acidosis-induced osteoclast adhesion by the RGD peptide treatment. Osteoclasts were resuspended in HEPES-buffered medium containing the GRGDSP or GRGESP peptide (control) at a final concentration of 100 μg/ml and plated on a 48-well culture plate coated with vitronectin. Adhesion was assessed 1 h after inoculation. (C) Inhibition of acidosis-induced osteoclast migration by the RGD peptide treatment. Osteoclasts resuspended in HEPES-buffered medium containing the GRGDSP or GRGESP peptide (100 μg/ml) were applied to the migration assay. All data represent mean ± SD. $^{**}P < 0.01$.

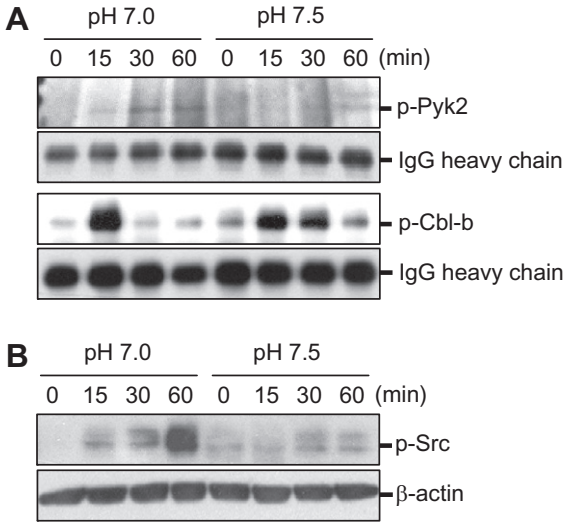

Fig. 4. Acidic pH stimulates signals involved in osteoclast adhesion and migration. (A) Osteoclasts were resuspended in HEPES-buffered serum-free medium, seeded on a 60 mm culture dish coated with vitronectin, and incubated to allow adhesion for the indicated times. The active forms (phosphorylation) of Pyk2, Cbl-b (A), and Src (B) were analyzed by immunoblotting.

[18,19], but Akt activation was irrelevant to alterations in pH (data not shown). These combined results indicate that the expression of bone matrix protein osteopontin was upregulated under low pH condition during osteoclastogenesis, which in turn induced an increase in osteoclast adhesion, migration, and survival via binding

of the extracellular RGD motif-harboring matrix to the cell surface integrin receptor and its cognate signal.

Previous studies have independently reported that acidic pH stimulates osteoclast function [17,21] and strengthens the cell surface integrin αvβ3-extracelluar matrix ligand bond in CHO cells [22]. Additionally, we found that acidic extracellular pH upregulated osteopontin and activated adhesive and migratory signals. Acid-induced osteoclast adhesion and migration depends on the interaction of the RGD motif-containing matrix protein and osteoclast surface integrins. Based on our and other previous reports, we propose the following possibilities: (i) systemic acidosis under pathological conditions may facilitate osteoclast formation, adhesion, migration, and survival by intensifying the integrin αvβ3-matrix protein interaction and by propagating stimulatory signals corresponding to each stage, thereby accelerating osteoclast function and osteoporotic bone loss. (ii) Localized acidic pH at the lacuna formed during normal osteoclast-mediated bone resorption may help to facilitate the tight sealing of the surrounding plasma membrane and bone surface matrix protein and promote osteoclastic bone resorption by direct bone mineral dissolution and by synergic activation of mature osteoclast migration and survival.

Our findings provide evidence that fine tuning of systemic and local physiological pH within a narrow range is essential for maintaining bone remodeling and a healthy skeleton. In the future, targeted factors regulated by pH dependence will be applied to therapeutic drug designs to treat acidosis-induced osteoporotic bone defects.

Acknowledgments

This work was supported by Grants from the Korea Science and Engineering Foundation (Nos. 2009-0077641, 2010-0012161, and 2011-0006178, to D.J.) and the National Research Foundation (No. 351-2009-1-E00021, to J.M.K.).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.12.149.

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