SPANDIDOS PUBLICATIONS

Vacuolar-type H$^+$-ATPase-mediated acidosis promotes *in vitro* osteoclastogenesis via modulation of cell migration

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Abstract. Localized acidification of the osteoclast-bone interface is driven by a vacuolar-type H$^+$-ATPase (V-ATPase) in the plasma membrane in a process thought to be associated with bone resorption. The present study investigated the mechanism underlying the roles of V-ATPase-induced acidosis in osteoclastogenesis. Active proton pumping due to increased V-ATPase activity during RANKL-induced osteoclastogenesis induced intracellular and extracellular acidification of osteoclast precursors. Subsequent analysis revealed blockage of extracellular acidification and induction of intracellular acidification by bafilomycin A1, a specific inhibitor of V-ATPase, indicating that extracellular acidification is mostly induced by V-ATPase-mediated proton pumping into extracellular space. Low-pH media controlled by HEPES-buffered conditions to mimic metabolic acidosis led to synergistic activation of RANKL-stimulated signals, including mitogen-activated protein kinases and transcription factor NF-κB, resulting in enhanced osteoclastogenesis. Low-pH media also upregulated the expression of osteopontin secreted into extracellular space, which is required for cell migration by binding to cell surface integrin αvß3. Osteoclast precursor migration was significantly inhibited by treatment of antibodies to integrin αvß3, resulting in the retardation of osteoclastogenesis. Taken together, these findings indicate that V-ATPase-driven acidosis modulates osteoclastogenesis.

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Introduction

Bone remodeling is achieved by the opposing actions of bone-forming osteoblasts and bone-resorbing osteoclasts (OCs). Receptor activator of NF-κB ligand (RANKL)-induced OCs are derived from the monocyte-macrophage lineage through a multistep process of cell adhesion, proliferation, migration, cell-cell contact, and terminal fusion of mononuclear cells to form multi-nuclear cells (1,2).

A stable physiological pH is essential for cell function and survival in mammals; intracellular pH (pH*i*) must be maintained within a narrow range to finely tune normal cellular metabolism. Chronic metabolic acidosis, induced when dietary intake contains more acid than base precursors, is associated with a poorly characterized metabolic bone loss, osteoporosis (3,4). In addition, it has been demonstrated that OC activity is highly dependent on extracellular pH (pH*e*), with increased activity under acidic conditions and decreased activity under alkaline conditions (5,6).

OCs exist on the bone surface and form a specialized ruffled border comprising a tightly-sealed compartment of plasma membrane in their bone-resorbing microenvironment (7). Several lines of evidence suggest that the primary cellular mechanism responsible for acidification of OCs on the ruffled border membrane is a vacuolar-type H$^+$-ATPase (V-ATPase) that actively pumps protons into the extracellular environment (8-11). This acidification of the localized compartment accelerates the solubilization of bone minerals and the degradation of bone matrix protein by lysosomal cysteine proteinases such as cathepsins secreted by the OCs (12-14). One cause of acidosis in OCs is carbonyl anhydrase II (CA II), which is both characteristic of OC differentiation and essential to OC bone-resorbing activity (11). A CO_2 hydration reaction by CA II generates carbonic acid, which dissociates to bicarbonate and protons at a physiological pH. This is the primary source of protons pumped into the resorption space by the V-ATPase. CA II inhibition is induced by an increase in pH*i*, which also decreases OC formation

and bone resorption (15). pH*i* regulation by CA II thus plays a critical role in the modulation of OC differentiation and function. Moreover, the targeted disruption of V-ATPase results in severe osteopetrosis due to a loss of OC-mediated extracellular acidification (16). Although a growing number of studies has implicated an interdependence of V-ATPase and CA II and a role for both in regulating bone resorption, the mechanism underlying the stimulatory effect of V-ATPase-induced acidosis on OC differentiation has not yet been elucidated.

In this study, we investigated changes in V-ATPase activity during OC differentiation and the effects of medium pH on OC differentiation. Our findings indicate that the activity of V-ATPase gradually increases during OC differentiation, and that a decrease in medium pH increases OC differentiation through a synergistic strengthening of RANKL-induced signaling activation and the enhanced OC precursor migration.

Materials and methods

Medium preparation. To test the effect of culture medium pH on osteoclastogenesis, α-MEM (Invitrogen, Carlsbad, CA) with HEPES (10 or 50 mM) in place of $NaHCO_3$ was adjusted to pH 7.0, 7.5, or 8.0 with 1 N NaOH.

Cell culture and differentiation. The murine monocytic RAW264.7 cell line, which is capable of differentiating into OCs upon stimulation with RANKL, was maintained under a humidified atmosphere of 5% CO_2 at 37˚C in DMEM (Invitrogen) buffered with $NaHCO_3$ and supplemented with antibiotics and 10% FBS. To examine the effect of pH alteration on OC differentiation, cells exposed to bicarbonate- and HEPES-buffered medium were grown in a humidified atmosphere with or without CO_2, respectively. To facilitate differentiation of RAW264.7 cells into OCs, cells were incubated with RANKL (100 ng/ml) for 4 days. Fresh media containing RANKL were applied at day 2. To differentiate the bone marrow-derived monocytes (BMMs) from the tibia and femur of C57BL/6 female mice into OCs (17), BMMs were incubated with macrophage colony-stimulating factor (M-CSF, 5 ng/ml; Genetics Institute, Cambridge, MA) in α-MEM containing 10% FBS for 12 h. Floating cells were cultured in α-MEM containing M-CSF (30 ng/ml) for 4 days to form OC precursors. To generate OCs, cells were further cultured with M-CSF (30 ng/ml) and RANKL (100 ng/ml) for 4 days. Fresh media containing M-CSF and RANKL were resupplied at day 2.

V-ATPase activity. Cell culture dishes were washed with 50 mM Tris-HCl (pH 7.0) once a day following OC differentiation and were stored at -70˚C until used. Cells were suspended in a buffer [20 mM Tris-HCl, pH 7.0, 1 mM EDTA, 1 mM DTT, 0.6% CHAPS, 1.5% nonyl-ß-D-glucopyranoside (Sigma-Aldrich, St. Louis, MO) and 10% glycerol], incubated for 10 min at 4˚C on a rotator, and centrifuged at 15,000 x g for 1 h. The supernatant containing the cytosolic fraction and solubilized membrane was immunoprecipitated by using Immunopure® Immobilized protein A/G beads (Pierce, Rockford, IL) cross-linked to antibody against the V-ATPase E subunit (Santa Cruz Biotechnologies Inc., Santa Cruz, CA) overnight at 4˚C on a rocker. After centrifugation, V-ATPase-antibody-protein A/G complexes were washed with a buffer (20 mM Tris-HCl, pH 7.5, 5 mM sodium azide, 0.1% CHAPS, and 0.1% nonyl-ß-D-glucopyranoside) three times. Immunoprecipitates were used to measure V-ATPase activity as described (18).

Tartrate resistant acid phosphatase (TRAP) staining and fusion efficiency. After differentiation into OCs, cells were fixed with 10% formalin for 10 min and rinsed with a 1:1 mixture of ethanol and acetone. Cells were stained for 10 min with Fast Red Violet LB salt (500 μg/ml, Sigma-Aldrich) dissolved in tartrate resistant acid phosphatase (TRAP) buffer (120 mM sodium acetate, 66 mM tartrate, and 100 μg/ml naphthol AS phosphate, pH 5.2). TRAP-positive multi-nucleated cells [TRAP(+) MNCs] with three or more nuclei per cell were counted as OCs.

Migration assay. Cell migration was determined using transwell chambers with polycarbonate membranes (pore size, 8 μm; Corning Inc., NY), with some modifications to the protocol described previously (19). To test the effect of medium pH on cell migration, RAW264.7 OC precursors were suspended in bicarbonate- or HEPES-buffered serum-free α-MEM [0.5% FBS and 0.1% bovine serum albumin (BSA) neutralized to ~pH 7.5 with 1 N NaOH], adjusted to pH 7.5 or 8.0 and seeded in the upper chamber of a transwell coated or not with osteopontin (OPN, 100 ng/ml; Sigma-Aldrich) at 4˚C overnight. Cells were allowed to migrate for 24 h under a CO_2-supplied or CO_2-free atmosphere. Cells were then fixed with 10% formalin for 10 min and stained with 1% crystal violet dissolved in 10% formalin for 5 min. Cells that had not migrated through the filter from the upper to the lower chamber were removed with a cotton swab, viewed under a light microscope, and counted.

Visualization of pHi by DAMP staining. Cells re-suspended in bicarbonate-buffered culture media were allowed to attach to glass coverslips for 6 h. After the replacement of HEPES-buffered culture media and exposure to CO_2-free conditions, cells were stained with 60 μM 3-(2,4-dinitroanilino)-3'-amino-N-methyldipropylamine (DAMP, Invitrogen) for 30 min, washed with ice-cold PBS, fixed with 3.7% formalin for 20 min, and permeabilized in methanol for 5 min at -20˚C. After rinsing in PBS, cells were blocked with PBS containing 1% BSA for 30 min, incubated overnight with mouse anti-dinitrophenol antibodies (Sigma-Aldrich) at 4˚C, and stained with FITC-conjugated goat anti-mouse IgG (Becton-Dickinson, San Jose, CA) for 2 h at room temperature. After washing three times, samples were mounted on glass slides and viewed under a confocal laser scanning microscope equipped with fluorescence (LSM 5 PASCAL; Carl Zeiss, Oberkochen, Germany).

Western blot analysis. Cells were stimulated with RANKL (100 ng/ml) for 10 min, washed once with ice-cold PBS, and lysed in a cell lysis buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM Na_3VO_4, 1 mM glycerol phosphate, 0.5% Na-deoxycholate, 1 mM EDTA, 1% Nonidet P-40,

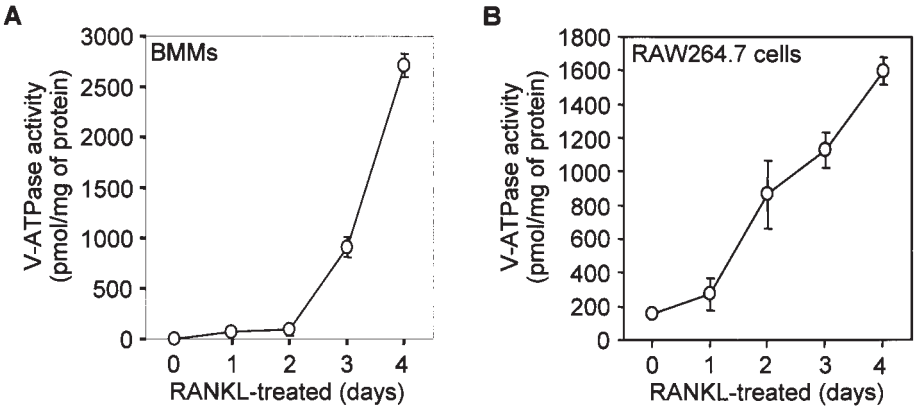

Figure 1. Changes in V-ATPase activity during OC differentiation. (A) V-ATPase activity during differentiation of BMMs into OCs. BMMs ($5x10^6$ cells/100-mm dish) were cultured with M-CSF in α-MEM for 3 days and further incubated with M-CSF and RANKL for 4 days to generate OCs. (B) V-ATPase activity during differentiation of RAW264.7 cells into OCs. RAW264.7 cells ($1x10^6$ cells/100-mm dish) were cultured with RANKL for 4 days. Cells were then collected at 1-day intervals. Next immunoprecipitation of the V-ATPase was performed by incubation of cell lysates with protein A linked to the antibody specific to the V-ATPase E subunit. V-ATPase activity was determined by the liberation of orthophosphate from ATP.

0.1% SDS, 1 mM PMSF, and 1X protease inhibitor cocktail (Roche, Mannheim, Germany). After centrifugation, the supernatant was resolved by 10% SDS-PAGE, electroblotted onto an Immobilon-P membrane (Millipore, Bedford, MA), and probed with primary antibodies to MAPKs [extracellular signal-regulated kinase (ERK), phospho-ERK, c-Jun NH_2-terminal kinase (JNK), phospho-JNK, p38, and phospho-p38] (Cell Signaling Technology, Beverly, MA), IkBα (Cell Signaling Technology), osteopontin (OPN; R&D Systems, Minneapolis, MN), and ß-actin (Sigma-Aldrich). All blots were detected with HRP-conjugated secondary antibodies and enhanced chemiluminescence reagents (Lab Frontier, Seoul, Korea).

RT-PCR. Total RNA was extracted from cells using TRI-reagent (Molecular Research Center, Cincinnati, OH) and was then reverse transcribed to cDNA. This cDNA was used as a template for PCR amplification (25 cycles of denaturation at 94˚C for 1 min, annealing at 57˚C for 30 sec, and extension at 72˚C for 1 min). Primers were synthesized for OPN (20) and hypoxanthine guanine phosphoribosyl transferase (HPRT) (21). PCR products were resolved on a 1% agarose electrophoretic gel, stained with ethidium bromide, and visualized under UV illumination.

Statistical analysis. Data were evaluated by analysis of variance (ANOVA) using the STATISTICA 6.0 software package. When significant differences were found, pairwise comparisons were performed using Scheffe's adjustment. Differences were considered statistically significant for $P<0.05$.

Results

Changes in V-ATPase activity during OC differentiation. OC V-ATPase is a multi-subunit complex with two distinct functional domains, including a catalytic V_1 domain and a proton-pumping V_0 domain (22). This complex is present in the plasma membrane and cellular organelle membranes, including those surrounding the endosome, Golgi apparatus, and lysosome (23). There is also marked accumulation of V-ATPase on the ruffled border membrane of OCs, where the complex pumps protons into an extracellular resorption area and results in the dissolution of hydroxyapatite mineral and degradation of bone matrix. Although cumulative data suggest that specific subunit expression of V-ATPase during 1,25-dihydroxyvitamin D_3-induced mouse bone marrow cultures is upregulated (24), and V-ATPase-mediated acidosis is related to bone metabolism (8-11), the trends of V-ATPase activity during OC differentiation remain unclear. Consequently, we assessed whether V-ATPase activity was altered during RANKL-induced OC differentiation. We found that V-ATPase activity was increased gradually by RANKL in a time-dependent manner (Fig. 1). Several reports have suggested that this activity is regulated by gene expression (24-26), the functional combination of each subunit in a multiple subunit complex (27,28), and the external-environment pH (9). Although the precise mechanism leading to enhanced V-ATPase activity during osteoclastogenesis remains to be elucidated, our initial findings led us to consider the possibility that V-ATPase is implicated in osteoclastogenesis.

Changes in pHi and pHe by RANKL or bafilomycin A1. Because V-ATPase pumps protons into the extracellular environment and V-ATPase activity is increased during OC differentiation, we proceeded to test whether V-ATPase-mediated intracellular and/or extracellular acidosis is/are induced during OC differentiation. To address this, we reconsidered a buffering capacity of medium pH to observe pH changes within a narrow range. Previous study has shown that pH*e* is lowered more by metabolic acidosis than by respiratory acidosis (29). Metabolic acidosis and respiratory acidosis were regulated by the concentration of bicarbonate and partial pressure of carbon dioxide, respectively (30). In this study, we observed that the pH of HEPES-buffered media without bicarbonate and CO_2 was constant while the pH of bicarbonate-buffered media containing CO_2 was not, consistent

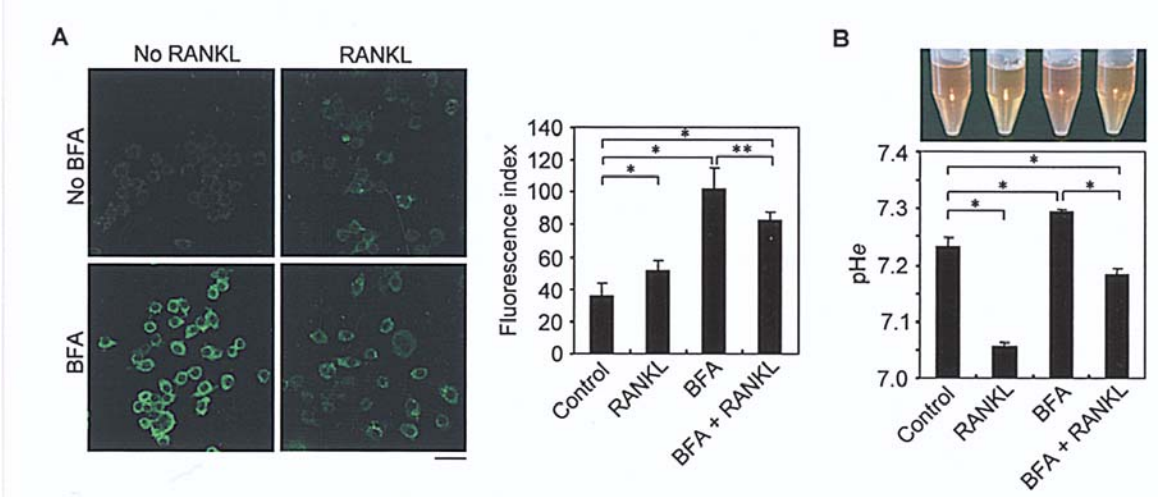

Figure 2. Changes in pH*i* and pH*e* by RANKL, BFA or both. (A) pH*i* changes using DAMP staining. RAW264.7 cells were seeded on a 60-mm culture dish ($4x10^5$ cells/4-ml dish), adapted for 6 h, replenished with 10 mM HEPES-buffered α-MEM at pH 7.5, transferred to a CO_2-free atmosphere to mimic metabolic acidosis, and then treated with 2 nM BFA for 30 min followed by incubation with RANKL for 2 days. Changes in pH*i* were determined by measuring dinitrophenol-fluorescence, reflecting the extent of acidosis. The fluorescence index was represented as the collected values of fluorescence intensity for total scan time of 5 sec. Data are expressed as mean ± SD (n=6); $^*P<0.01$ and $^{**}P<0.05$. Bar, 50 μm. (B) pH*e* changes in culture media. After experiments were performed as described in (A), the pH*e* of culture medium was monitored directly with a pH meter (Thermo Orion Model 420), and medium color was photographed. Data are expressed as mean ± SD (n=3); $^*P<0.01$ and $^{**}P<0.05$.

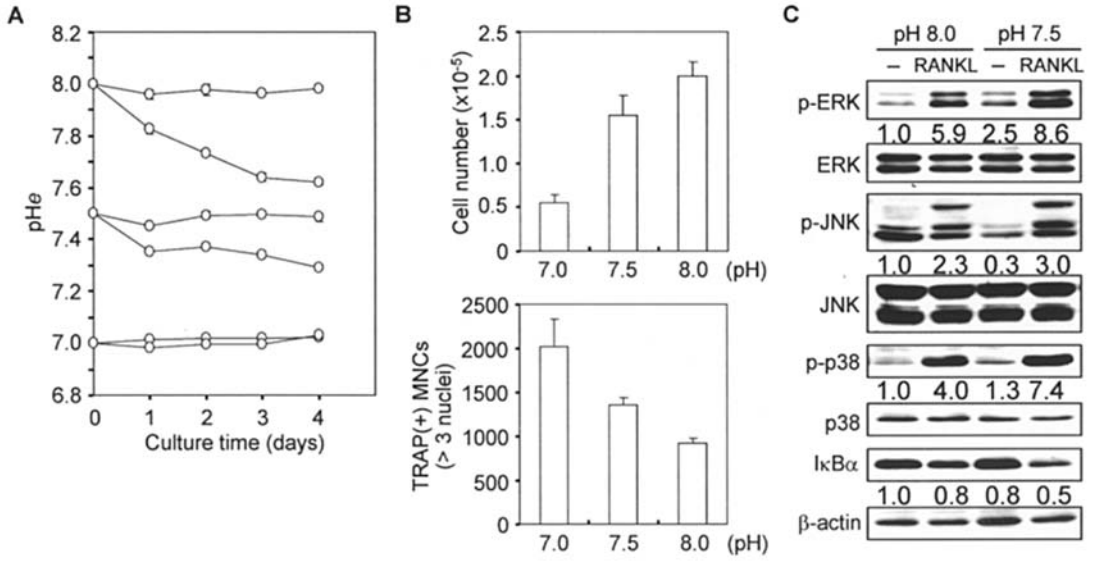

Figure 3. Stimulatory effect of low-pH media on osteoclastogenesis. (A) Profile of pH*e* under CO_2-free conditions. RAW264.7 cells were plated on a 60-mm culture dish at a density of $2x10^5$ cells/4-ml dish, adapted for 6 h, and replenished with 50 mM HEPES-buffered α-MEM at pH 8.0, 7.5, or 7.0. After culture dishes were transferred to CO_2-free conditions, the change in pH*e* of the culture medium at the indicated times was monitored directly with a pH meter. Data are expressed as mean ± SD (n=3). ○, medium alone; and •, medium with cell. (B) Effect of different pHs on osteoclastogenesis. After transfer of the cells treated with RANKL to a CO_2-free incubator, the number of OCs with more than three nuclei was analyzed at day 4. Data are expressed as mean ± SD (n=4). (C) Effect of media pH on RANKL-induced signaling. Cells ($1x10^6$ cells/dish) were seeded on a 100-mm culture dish and maintained overnight in 50 mM HEPES-buffered media (pH 8.0 or 7.5). This was followed by stimulation with RANKL for 10 min. Cytosolic fractions were subjected to Western blot analysis with specific antibodies.

with several previous results (29-31). The pH of bicarbonate-buffered α-MEM also showed a protean tendency during a 48-h incubation under CO_2 supply; after equilibration, the pH of 7.5 and 7.0 changed to ~7.42 and 7.38, respectively. The pH of HEPES-buffered α-MEM under CO_2-free conditions, in contrast, remained almost unchanged (pH 7.5 and 7.0 initially, pH 7.47 and 7.02 after equilibration). Based on these observations, we used metabolic acidosis in the present study as a reproducible pH model system to determine the changes of pH*i* and pH*e* in RANKL-induced osteoclastogenesis.

In order to observe the acidic cytosolic compartment, we performed immunocytochemical staining with DAMP, which enables the detection of acidic parts (32), under HEPES-buffered medium in the absence of CO_2. Treatment with RANKL or bafilomycin A1 (BFA), a specific inhibitor of V-

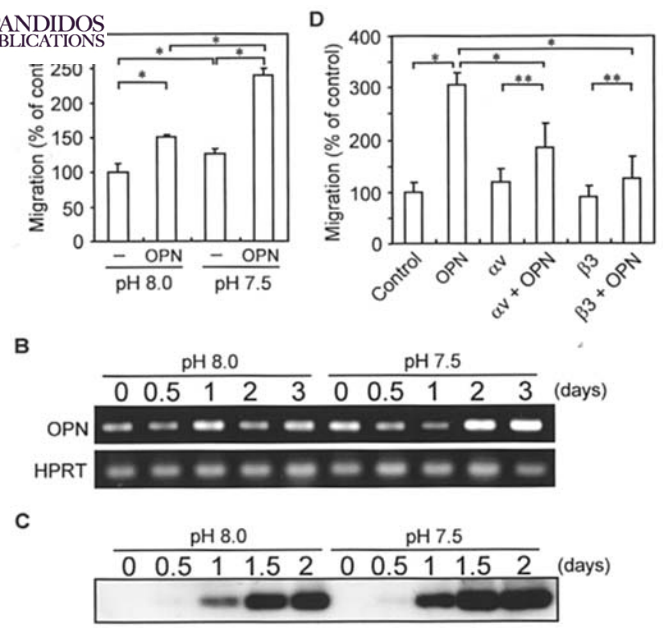

Figure 4. Stimulatory effect of low-pH media on cell migration and OPN gene expression. (A) Effect of media pH on OPN-induced migration. The migration of RAW264.7 OC precursors was assessed in 50 mM HEPES-buffered serum-free media adjusted to pH 8.0 or 7.5. After OC precursors ($1x10^4$ cells/well) were seeded into the upper chamber of a transwell coated or not with OPN and incubated for 24 h, the extent of migration was quantified by counting all cells that had migrated through the filter as described in the Materials and methods section. Data are expressed as mean ± SD (n=8); *P<0.01. (B) mRNA level for OPN. After cells were exposed to HEPES-buffered media under CO_2-free conditions, the mRNA level for OPN and HPRT at the indicated times was determined by RT-PCR. Co-amplification of the HPRT gene was performed in order to confirm the relative expression level. (C) The expression level of secreted OPN in culture media. Cells ($5x10^5$ cells/4-ml dish) were cultured with HEPES-buffered media containing 0.5% FBS under CO_2-free conditions. Culture media were stored at -20˚C until used at the indicated times. Media (30 μl) were fractionated by SDS-PAGE on a 12% gel and then subjected to Western blot analysis using specific antibodies to OPN. (D) Role of cell surface integrin αvß3 in migration. A mixture of OC precursors suspended in medium, pH 7.5, and antibodies (7.5 μg/ml) to integrin subunit αv or ß3 was preincubated in the tube for 30 min and then loaded on the upper chamber of transwells coated with OPN. Cells were allowed to migrate for 24 h. Data are expressed as mean ± SD (n=8); *P<0.01 and **P<0.05.

ATPase (9), in OC-precursor RAW264.7 cells induced intracellular acidification (Fig. 2A). We also found that RANKL treatment slightly reduced BFA-induced intracellular acidification (Fig. 2A, column 3 vs 4). This phenomenon might have been caused by active proton secretion from cytoplasmic compartments that accompany increased V-ATPase activity during RANKL-induced osteoclastogenesis.

To directly measure the pH of extracellular compartments, RAW264.7 cells were cultured in HEPES-buffered media with RANKL, BFA, both, or neither. Alterations in culture medium pH*e* were monitored directly by color change and a pH meter two days after the transfer of the cells to CO_2-free conditions (Fig. 2B). The pH*e* of RANKL-treated cells was significantly lower (7.06±0.01) than that of untreated control cells (7.23±0.02). In contrast, the BFA treatment increased pH*e* to 7.29±0.01. BFA initially caused an increase in intracellular acidification, after which a decrease in extracellular acidification was observed. In addition, changes in pH, as mentioned above, showed a similar pattern in OC precursors derived from BMMs (data not shown). These findings suggest that acidification in the extracellular environment upon osteoclastogenesis arises from the increased activity of V-ATPase in the plasma membrane.

Stimulatory effects of metabolic acidosis on RANKL-induced signaling and osteoclastogenesis. We next hypothesized that extracellular acidification may be related to RANKL-induced OC differentiation. We tested both the effects of different pHs (pH 7.5 vs 8.0) and different concentrations of HEPES (10 vs 50 mM) in a buffer on cell growth and osteoclastogenesis. Relatively low pHs and low concentrations enhanced osteoclastogenesis, but also retarded cell growth (data not shown). During incubation under CO_2-free conditions, the pH (7.0, 7.5, or 8.0) of HEPES-buffered medium alone was constant, while the pH (7.5 or 8.0) of culture media with cells was decreased in a time-dependent fashion (Fig. 3A). Although the pH of culture media at 7.0 was not changed by causing the arrest of cell growth, the number of TRAP(+) MNCs and their size increased at a lower pH compared to a higher pH, indicating that a relatively low pH promotes osteoclastogenesis (Fig. 3B). Our findings indicate that a buffering capacity of medium pH regulates osteoclastogenesis, with reduced pH and buffering capacity resulting in increased osteoclastogenesis.

To evaluate the mechanism underlying acidosis-induced osteoclastogenesis, we reconsidered the cellular growth and the tendency of acidosis with decreasing pH. To do this, we adopted 50 mM HEPES-buffered media at pH 8.0 and 7.5 for the following trials. Since low pH regulates OC differentiation, we predicted that medium pH would influence RANKL-induced signals. Osteoclastogenesis-related signaling pathways in the presence of RANKL were upregulated under the conditions of low medium pH (Fig. 3C). These findings suggest that RANKL and a relatively low pH may act synergistically to induce RANKL-mediated signal activation.

Effects of acidic conditions on OC precursor migration. As osteoclastogenesis is a multistep process including cell adhesion, migration, and fusion-to form TRAP(+) MNCs, we could not exclude cell adhesion and migration as causes of the stimulatory effect of low pH on osteoclastogenesis. However, we first excluded cell adhesion because there had been the adaptation introduced by adhering the cells to the culture dish prior to RANKL-induced OC differentiation.

Several lines of evidence have suggested that osteoclastic bone resorption occurs by serial coordination of adhesion to bone matrix (33), secretion of acid and proteases to the ruffle border, and active OC migration along the bone surface (19,33-35). Moreover, OPN which is a secreting protein within extracellular space and integrin αvß3 which exists in the plasma membrane have been implicated in the regulation of cell adhesion and migration in OC precursors and mature OCs (19,33-35). We found that a decreasing medium pH stimulated OPN-induced OC precursor migration (Fig. 4A). We also observed that exposure to medium at pH 7.5 showed high levels of OPN mRNA (Fig. 4B) and an increased pattern

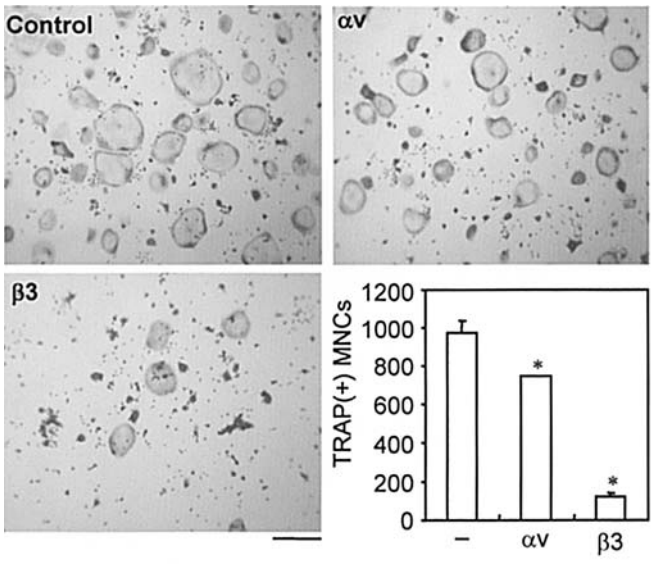

Figure 5. Role of cell surface integrin αvß3 in OC differentiation. After RAW264.7 cells were adapted in a culture dish to allow the adhesion, cells were further cultured in the presence of RANKL with 1.25 μg/ml non-immunized IgG, 1.25 μg/ml integrin subunit αv IgG or 1.25 μg/ml integrin subunit ß3 IgG for 4 days. After fixing and staining cells for TRAP, the number of TRAP(+) MNCs were counted as OCs. Data are expressed as mean ± SD (n=3); $^*P<0.01$ vs control. Bar, 200 μm.

of OPN protein in the culture media of RANKL-stimulated cells compared with medium at pH 8.0 (Fig. 4C). It is possible that an increased interaction of OPN with integrin αvß3 results in this stimulated migration of OC precursors. Treatment with antibodies for αvß3 blocked cell migration towards OPN (Fig. 4D). We also observed that the addition of antibodies specific to integrin subunits αv and ß3 inhibited OC differentiation in RANKL-stimulated cultures (Fig. 5). Antibody-blocking studies suggested that integrin αvß3 is capable of interacting with OPN and is essential for osteoclastogenesis. Thus, relatively low pH conditions likely contribute to upregulated gene expression of OC precursor migration-related OPN and result in enhanced cell migration and osteoclastogenesis.

Discussion

Maintenance of a stable physiological pH is critical to normal cell function and to cell survival in mammals. It has been reported that metabolic acidosis leads to an increase in osteoclastic activity, which in turn results in bone loss (3,4). More specifically, proton pumping by V-ATPase acidifies the ruffled border environment of the OC-bone interface, and this acidification dissolves bone minerals and degrades bone matrix through the action of cysteine proteases such as cathepsin K (8-14). The effect of V-ATPases on metabolic acidosis is not fully understood, however, and neither are the molecular mechanisms underlying the action of V-ATPase-mediated acidosis on osteoclastogenesis. The data presented in this study provide the characterization of the contributing effects of low pH to osteoclastogenesis.

Cells constantly secrete protons across their plasma membranes into the extracellular environment in order to maintain an acid-base balance within the narrow physiological range appropriate for cellular function. In mammals, systemic pH has been found to be reduced by either a decrease in bicarbonate concentration (metabolic acidosis) or an increase in the partial pressure of carbon dioxide (respiratory acidosis) (30). The acidosis in OCs can be generated by RANKL-mediated metabolic stimulation via several mechanisms: i) the high number of mitochondria in OCs reflects high ATP turnover and results in proton generation as a byproduct of heightened metabolism (8); ii) CA II, initially expressed at a low level in OC precursors and later upregulated during osteoclastogenesis, facilitates the supply of protons to V-ATPase by promoting the hydration of CO_2 and generating carbonic acid (11); and iii) we observed that glucose and ATP consumption were increased during RANKL-dependent osteoclastogenesis (unpublished data). These findings indicate that OCs have high biosynthetic and metabolic activities that, among other functions, contribute to acidosis. When bone is cultured under metabolic acidosis, there is typically more bone loss than when cultured under respiratory acidosis. It has been reported that bone loss by metabolic acidosis is caused by a reciprocal suppression of osteoblastic bone formation and stimulation of physiochemical mineral dissolution and osteoclastic bone resorption (3,6,36). Several mechanisms may underlie the stimulatory effect of acidosis on bone resorption: i) V-ATPase activity could be a function of transcriptional and translational efficiency and mRNA and protein stability (24-26); ii) after stimulation with developmental triggers, this activity could be controlled by the serial assembly of the multi-subunit V-ATPase complex (27,28); and iii) low pH could directly stimulate V-ATPase activity (9). Although the exact mechanisms underlying the regulation of V-ATPase activity remain unclear, we have now shown that a progressive increase of V-ATPase activity occurs during RANKL-induced OC differentiation and induces increased acidification of the intracellular and extracellular spaces of OC precursors.

While our findings suggest that V-ATPase-mediated acidosis synergistically promotes RANKL-induced OC differentiation, Li *et al* reported that in mice with a targeted deletion of the *Atp6i* gene, a putative OC-specific proton pump subunit resulted in osteopetrosis by the decreasing bone-resorbing function of OCs due to blockage of OC-mediated extracellular acidification, but not by blocking OC formation (16). Although the roles of V-ATPase-mediated acidosis in OC differentiation *in vivo* are unclear as yet, one possible interpretation is that acidosis in the micro-environment of OC precursors through the combination of the accumulated protons by metabolic acidosis which induce bone loss (3,30), and the active proton pumping into extracellular space by V-ATPase may synergistically facilitate OC differentiation.

The binding of RANKL to its receptor RANK propagates multiple pathways that activate MAPKs, ERK, JNK, p38, transcription factors NF-κB and AP-1, and calcineurin/NFAT. Several reports have suggested that metabolic acidosis decreases osteoblastic bone formation and increases osteoclastic bone resorption, resulting in osteoporosis (4,6,36). Recently, it was further reported that NFATc1 activation by acidosis in mature OCs stimulates bone resorption through

tion of calcineurin and the suppression of NFATc1 on (37). Metabolic acidosis upregulates the gene expression of RANKL in bone (38), which stimulates OC differentiation and bone resorption, and CA II and calcitonin receptor (39), which modulate resorption. Three trends were consistently observed in this study: i) the lowering of extracellular pH by acidic media in the absence of RANKL triggered slight signaling responses related to OC differentiation (Fig. 3C); ii) RANKL and metabolic acidosis synergistically activated multiple signaling cascades, including ERK, JNK, p38, and transcription factor NF-κB, essential to the stimulation of osteoclastogenesis; and iii) the combination of RANKL and low pH facilitates induction of the OC precursor migration-related gene, OPN, and CA II (data not shown), which provides the supply of protons to V-ATPase, in a synergistic manner. Low-pH media accelerated the expression of OPN secreted into medium, stimulating migration and thereby promoting osteoclastogenesis. Findings from our experiments and others have enabled us to propose a schematic model for the effects of low pH on osteoclastogenesis. Our model is as follows. Metabolic acidosis follows the increase in V-ATPase activity that arises from the interaction of RANKL with its receptor RANK during osteoclastogenesis and synergistically stimulates RANKL-induced signal responses and OC precursor migration-related OPN gene expression. This results in the enhanced formation of mature OCs through high migration.

Recent reports have proposed a wide variety of promising therapeutic molecules for treating osteoporosis. The therapeutic effects of these molecules are thought to arise from their influence on OC differentiation or modification of proteolytic enzymes involved in bone matrix dissolution. Candidate therapeutic molecules include osteoprotegerin, c-src, CA II, cathepsin K, V-ATPase, and integrin αvß3 (40). The inhibition of V-ATPase by treatment with BFA (10) and by antisense RNA and DNA molecules targeted against V-ATPase (41) reduced bone resorption. An OC-specific proton pump subunit-deficient system also resulted in osteopetrosis (16). A functional study at the cellular level of the key molecules of bone remodeling may lead to the discovery of novel drugs for treating osteoporosis. Summarizing, our findings point to the RANKL-mediated V-ATPase induction/V-ATPase-mediated acidosis/RANKL-dependent signals/migration pathway as a potential therapeutic target for reducing osteoclastogenesis and preventing bone loss.

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