Review

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Promotion of osteoclast differentiation and activation in spite of impeded osteoblast-lineage differentiation under acidosis: Effects of acidosis on bone metabolism

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Summary **The acidosis that accompanies many diseases and pathological conditions can promote osteoclast formation and activation. Acidosis mainly acts on the last phase of osteoclast formation to generate large osteoclasts and promote bone resorption. There are several acid-sensing mechanisms, among which transient receptor potential (TRP) channels and G protein-related receptors have been focused on. TRPV4 channels appear to be, at least partly, implicated in acidosis-promoted large osteoclast formation. Other TRP channels including TRPV1 and TRPV2 might be components of the acid-sensing machinery. Several reports suggest the involvement of ovarian cancer G protein-coupled receptor 1 (OGR1), a G-protein-related acid sensor, in receptor activator of nuclear factor kappa-B ligand (RANKL) expression via cyclooxygenase-2 (COX-2). On the other hand, acidosis impairs osteoblast differentiation, which is further impeded in the presence of inflammatory cytokines.**

Keywords: Acidosis, acid sensing, osteoclast, osteoblast, bone metabolism

1. Introduction

The acid-base balance in the body has vital effects on cellular functions, because the structure and function of proteins are strictly controlled by the proton concentrations in tissue fluid, therefore, it could broadly influence the activity of enzymes in bone-related cells, the activity of transcription factors and the structure of other proteins involved in bone metabolism. We will explain the widely varying effects of acidosis on bone metabolism, mainly focusing on the mechanism behind the formation and activation of large osteoclasts. We will also describe the inhibitory effects of acidosis on osteogenic-lineage populations. There have recently been reports regarding the candidates of acid-sensing machinery, to which we will refer. We will pick up key mechanisms, which may explain acidosis effects on the metabolism of the bone system.

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2. Systemic acidosis and local acidosis

The pH of blood is of great importance to the body. A change of more than ± 0.05 from the physiologically neutral pH 7.4, results in acidosis (pH 7.35 or less) and alkalosis (pH 7.45 or more). The pH balance of extracellular fluids, blood, lymph, and interstitial fluid, are primarily attributed to the equilibrium between the acid-base balance composed of carbon dioxide (CO_2) and sodium bicarbonate ($NaHCO_3$). This balance cannot be maintained when the functions of organs deteriorate. The inability of the lungs to properly expel CO_2 can lead to respiratory acidosis, while defects in the kidney's function to excrete protons result in the consumption of bicarbonate ions (HCO_3^-) by remaining acids, metabolic acidosis (*1,2*).

Although organ failure invites acidosis systemically, numerous conditions and diseases can induce acidosis locally, local acidosis. These include tumors, inflammation, injury, infections, bone fractures, ischemia, hypoxia, and retardation of metabolic waste. These conditions necessarily affect the function of

surrounding tissues. Acidosis has been reported to drive bone metabolism toward bone resorption (*3*). On the other hand, mild alkalosis promotes osteoblast differentiation (*4*).

3. Acidosis and bone resorption

Acidosis impedes bone formation and promotes Ca^{2+} excretion. Alkalosis-inducing bicarbonate ions (HCO_3^-) in drinking water are reported to suppress Ca^{2+} excretion, whereas acidosis-inducing ammonium chloride (NH_4Cl) promotes Ca^{2+} excretion (*5-7*). Foods rich in nonvolatile acid precursors, for example, meat containing phosphorous or sulfur, lower blood pH (*2*).

Calvarial cultures under acidic conditions exhibit osteoclast activity to form pits on the surface of calvariae (*8*). This was confirmed using osteoclasts recovered from cocultures of bone marrow cells and osteoblastic cells on a collagen gel in the presence of 1,25,dihydroxy vitamin D_3 (1,25 $(OH)_2$ VD_3) and prostaglandine E_2 (PGE_2) in a regular culture medium for one week. We can control pH of the culture systems by adding different amount of $NaHCO_3$ to the cultures at 5% CO_2. When cultures consisting of osteoclasts and their nursing osteoblastic cells were transferred onto dentine slices in media with different pH to test for the ability to form pits, osteoclasts in acidic media, at pH 7.0 and pH6.8, formed pits more than in a neutral medium at pH7.4, indicating that acidosis activates osteoclast resorption (Figure 1). Although acidosis is important for osteoclast activity to resorb bone, interaction with osteoblasts appears to be vital to the activity of osteoclasts. Whereas osteoclasts derived from bone marrow cells using soluble receptor activator of nuclear factor kappa-B ligand (RANKL) and macrophage colony stimulating factor (M-CSF) showed only weak resorptive activity, they exerted strong activity when osteoblasts activated with 1,25 $(OH)_2$ VD_3 and PGE_2 were added to the osteoclast cultures just before the pit formation assay on dentine slices (Figure 2).

In an *in vitro* cell system, intracellular Ca^{2+} elevation under acidosis activates calcineurin which activates NFATc1; NFATc1 moves to the nucleus, where it acts as a critical transcription factor for osteoclast activation. Acidosis also appears to inactivate several protein kinases

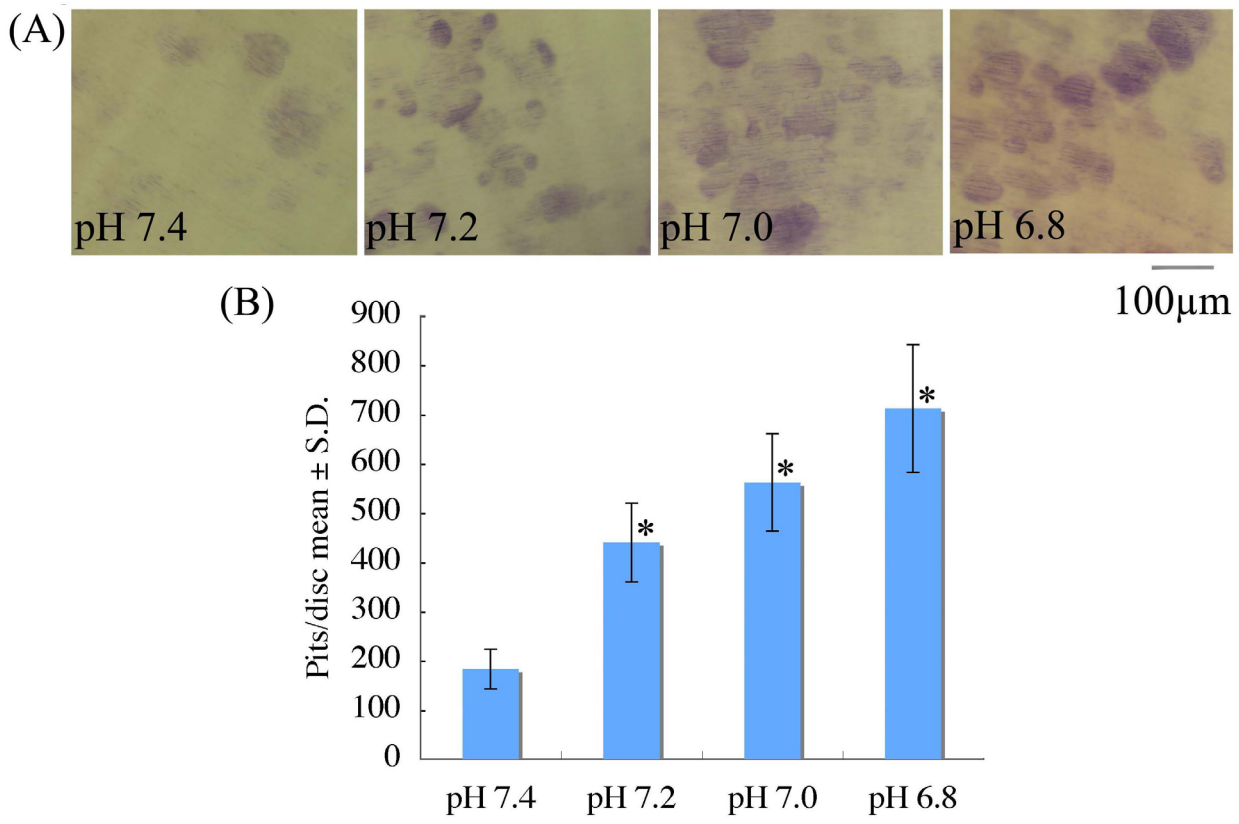

Figure 1. Acidosis promotes osteoclast activity. Mouse (male ddY) bone marrow cells were cocultured with TMS-12 cells, an osteoblastic cell line, on a type 1 collagen gel (2.4 mg/mL) in an α-MEM, 10% fetal calf serum, 10 nM 1,25 $(OH)_2VD_3$ and 1 μM PGE_2 at pH 7.3 for 7 days. After the digestion of the gel by 0.1% collagenase plus 0.2% dispase in α-MEM, cells were recovered, divided and suspended in media at pH 7.4, 7.2, 7.0, and 6.8. Cell suspensions were placed in 96-well plates with previously set discs of dentine slices at the bottom and kept in a humid atmosphere at 37ºC for 24 h. **(A)** Stains represent the pits. Bar under the photo represents 100 μm. **(B)** Pits of the resorbed trace on a disc were counted under a microscope after staining with 1% toluidine blue and scraping off the cells on the disc. Asterisks (*) represent differences that are statistically significant from control values at pH 7.4, $p < 0.05$, $n = 4$. All of the procedures for animal experiments were approved by the University Committee of Animal use.

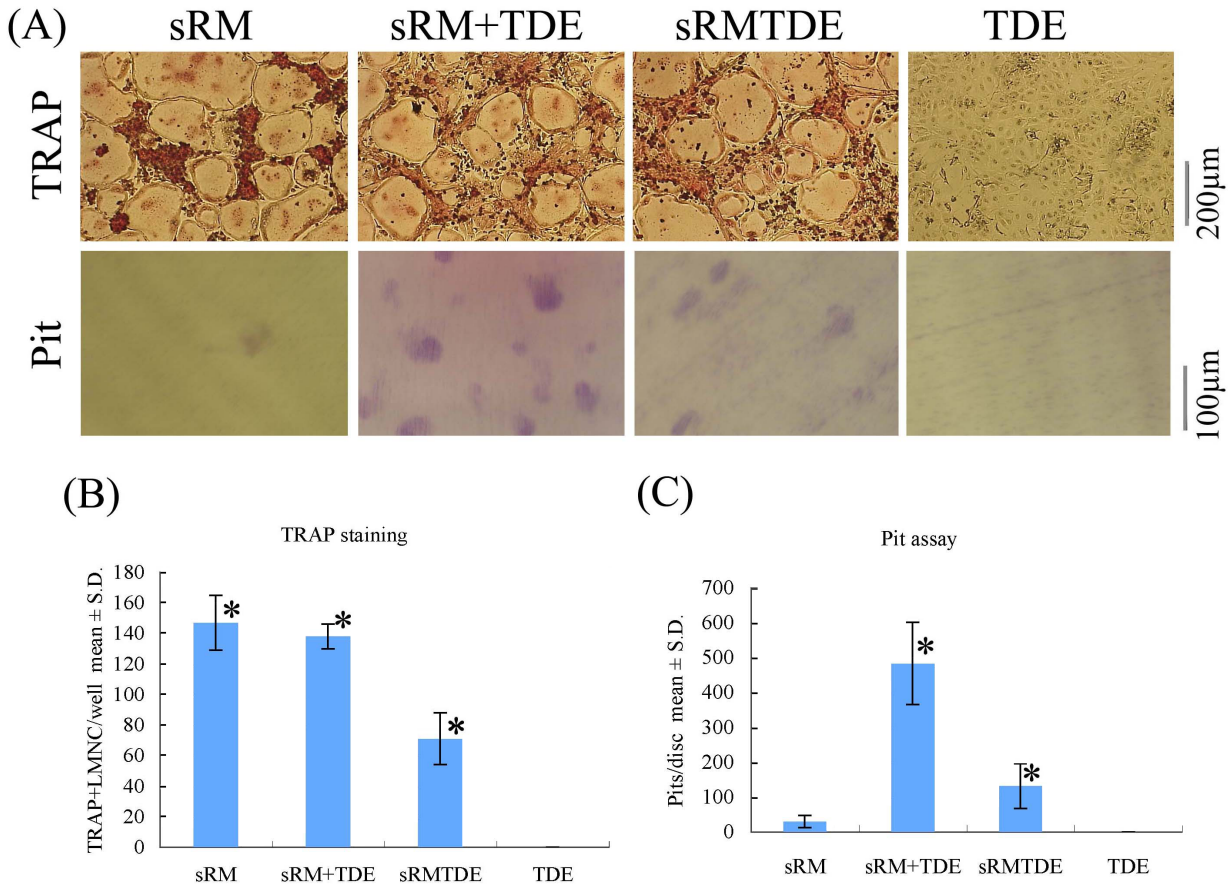

Figure 2. The cooperation between osteoclasts and osteoblasts appears to potentiate the bone resorptive activity of osteoclasts derived from bone marrow cells treated with soluble RANKL (sRANKL) and M-CSF. Mouse bone marrow cells cultured with 50 ng/mL sRANKL (sR) and 30 ng/mL M-CSF (M) in α-MEM-10% fetal calf serum at pH 7.3 for 3 days, sRM, were prepared. On the other hand, TMS-12 cells activated with 10 nM 1,25 $(OH)_2VD_3$(D) and 1 μM PGE2 (E) for 3 days, TDE. On day 3 sRM was combined with TDE, cultured in the presence of sRANKL and M-CSF at pH 6.8 for 24 h. **(A)** Tartarate-resistant acid phosphatase-positive large multinuclear cells (TRAP+LMNC: large osteoclasts) per well of 96-well plates. sRM cultures formed large osteoclasts. Although unstimulated TMS-12 cells and TMS-12 stimulated not enough suppressed large osteoclast formation from sRM, sRMTDE still stimulated pit formation. Bars under the photos of TRAP staining represents and those of pit formation assay represent 200 μm and 100 μm, respectively. **(B)** and **(C)**: graphical indications of TRAP staining and Pit formation assay, respectively. sRM cultures require activated TMS-12 to exert full resorptive activity. Asterisks (*) represent differences that are statistically significant from control values of sRM (cultures only containing activated TMS-12), $p < 0.05$, $n = 4$. TRAP + LMNC/large osteoclast: tertarate-resistant acid-phosphatase, a maker of osteoclasts, positive large multinuclear cells with 10 or more nuclei.

involved in the inactivation of NFATc1, maintaining NFATc1in the nucleus (*9*). Other than acidosis, PGE_2 appears to promote osteoclast formation in the absence of osteoblasts, implying the direct action of PGE_2 on RANKL-activated osteoclast lineage cells (*10*).

TRPV5, a member of a subfamily of the transient receptor potential (TRP) channel family, is reported to be indispensable for the differentiation of functional osteoclasts and for Ca^{2+} homeostasis in epithelial cells of the intestine and kidney. The loss of TRPV5 results in hypercalciuria, an increase in the numbers of less functional osteoclasts and loss of bone thickness (*11*). RANKL treatment induces intracellular Ca^{2+} elevation *via* TRPV5, but siRNA treatment to knockdown TRPV5 increased the bone resorptive activity of osteoclasts (*12*). Therefore, TRPV5 channels might function to monitor bone resorption. The effect of acidosis on TRPV5 channels remains to be clarified.

4. Acidosis and osteoclast formation

Acidosis also promotes the formation of osteoclasts, especially, large osteoclasts in several systems. It has been unclear where acidosis acts in the course of osteoclast formation, although there have been several reports that acidosis exerts promotive effects on osteoclastogenesis (*13,14*). We investigated this point to demonstrate that acidosis primarily acts on osteoclast differentiation in the last stage of the process, just before large-scale cell fusion (Figures 3A and 3B) (*15*). In a different coculture system, where osteoclasts were induced to differentiate with 1,25 $(OH)_2$ VD_3 and PGE_2 on a collagen gel in media with different pH, osteoclast formation was promoted at acidic pH and bone-resorbing activity of the cultures was also higher at lower pH than at a physiologically neutral pH7.4 (Figure 3C) (data not shown).

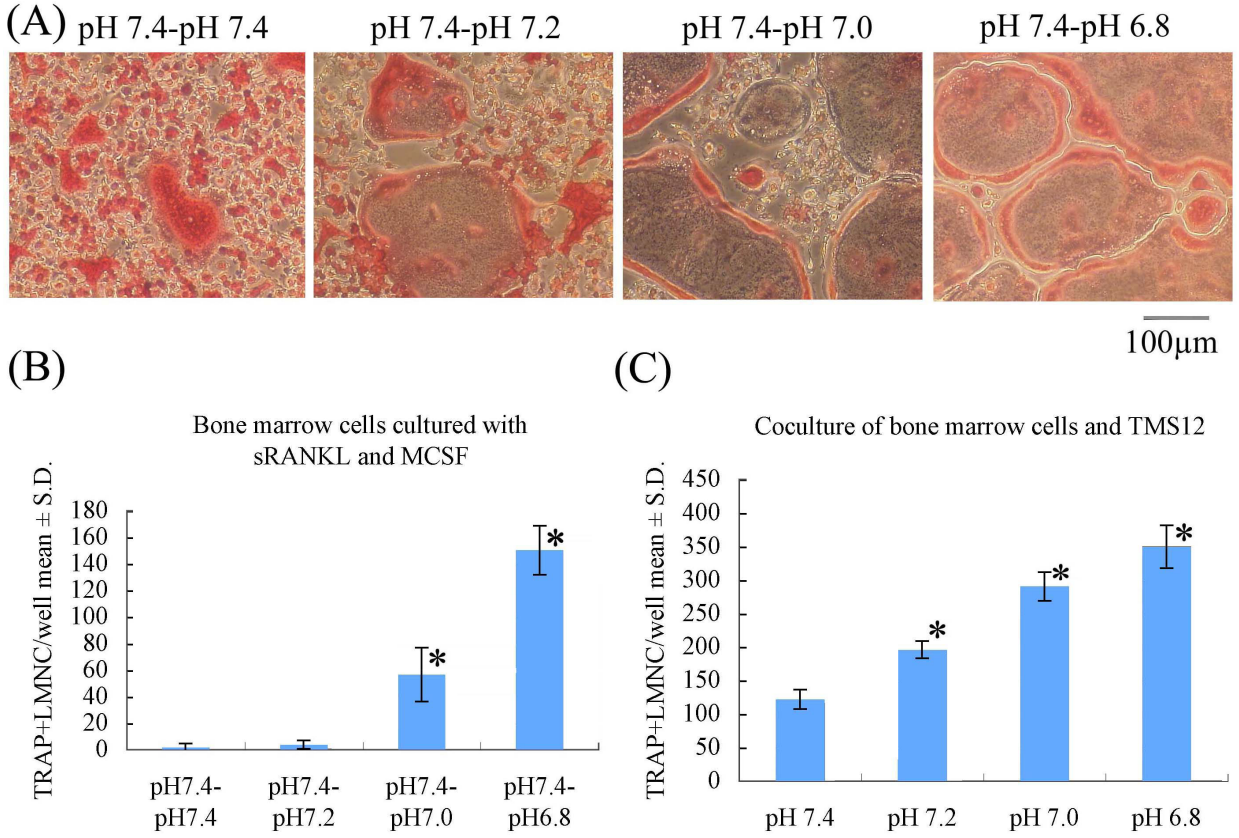

Figure 3. Acidosis acts on the last phase of large osteoclast formation in two different culture systems, monoculture and coculture on a collagen gel. (A) Mouse bone marrow cells were treated with sRANKL and M-CSF in α-MEM-10% fetal calf serum at pH 7.4 for 3 days. Cells were then cultured overnight in different media at pH 7.4, 7.2, 7.0, and 6.8. TRAP staining was performed on day 4. Photos represent TRAP + LMNC at 10 × 10 magnification. Bar under the photos represents 100 μm. **(B)** TRAP + LMNC numbers were counted after staining. Asterisks (*) represent differences that are statistically significant from control values at pH 7.4, $p < 0.05$, $n = 4$. **(C)** Mouse bone marrow cells were cocultured with TMS-12 cells, an osteoblastic cell line, on a type 1 collagen gel (2.4 mg/mL) in α-MEM, 10% fetal calf serum, 10 nM 1,25 $(OH)_2VD_3$ and 1 μM PGE_2 at pH 7.3 for 5 days. Then the cultures were divided into 4 groups; the media were replaced by a medium with pH 7.4, 7.2, 7.0, or 6.8; cultures were maintained for another 3 days, from day 5 to day 8; cells were recovered from the gel to test TRAP staining. Asterisks (*) represent differences that are statistically significant from control values at pH 7.4, $p < 0.05$, $n = 4$.

5. Acid-sensing machinery

Several types of acid-sensing systems have been reported, including members of acid-sensing G protein-related receptors, ovarian cancer G protein-coupled receptor 1 (OGR1) and T cell death-associated gene 8 (TDAG8) (*16-18*), of TRP, TRPV1 and TRPV4 (*19,20*), and of acid-sensing ion channels (ASICs) (*21*). TRPV1 and TRPV4 channels are permeable to several cations including Ca^{2+} and Na^+ and ASICs are specific for Na^+ ions. OGR1 was originally reported as a receptor for several lysophospholipids, lysophorylcholine, and sphyngosilphosphorylcholine (*16*), but later recognized as an acid-sensing receptor coupling with the Gq protein. On recognizing protons it changes molecule structure based on histidine residue-mediated transformation in an acidic environment. TDAG8 senses acid to move Gs, resulting in an elevation in the intracellular concentration of cyclic AMP (cAMP) (*18*).

The TRP family consists of cation-permeable channels with a variety of characteristics, differing in selectivity to cations including Ca^{2+} and Na^+. TRPV1, known as the receptor for capsaicin, is activated by acid. Several reports suggest the involvement of TRPV1 in osteoclastogenesis. Capsaicin, a TRPV1-specific agonist, enhanced osteoclast formation in murine bone marrow cultures treated with RANKL and M-CSF (*22*). On the other hand, capsazepine, a TRPV1-specific antagonist, suppressed osteoclast formation and the bone resorptive activity of osteoclasts (*23*). TRPV1-specific antagonists, capsazepin and 5-resiniferatoxin, also suppressed osteoblast differentiation. In our acidosis-promoted osteoclast formation system, capsaicin did not potentiate osteoclast formation, and AMG9810, a TRPV1-specific antagonist, did not inhibit osteoclast formation. The difference in systems used may cause different results.

TRPV4 was first reported as a channel sensing low osmolarity (*20*), but later found to be sensitive to mechanical stress (*24*). Vascular endothelial cells are known for their sensitivity to stretching, rotating

their orientation or spindle-shape perpendicular to the direction of stretch when scattered into elastic wells and cultured with cyclic stretch, which is attributed to TRPV4 (*25*). Several reports refer to knockout mice with depletion of TRPV4 (*26*). They are insensitive to the tail suspension test, which mimics microgravity (*27*). The mice also showed defects in osteoclast formation and activity, with large osteoclasts small in number (*28*). TRPV4 shows a weak response to acid (*29*), and is activated by src under acidotic conditions (*30*), implying that TRPV4 could be activated during acidosis.

TRPV4 channels are produced in the last phase of osteoclast differentiation, acting as Ca^{2+} channels to sustain the intracellular Ca^{2+} level for the maintenance of active NFATc1 (*28*). The precise control system for TRPV4 has recently been reported, with the calmodulin kinase system having important roles downstream of the channels (*31*).

Several membrane-derived arachidonic acid metabolites are known to activate TRPV4 (*20*). PGE_2 activates TRPV1 channels *via* PKC phospholyration in which EP1 is involved (*32*). PGE_2 potentiated osteoclast formation by soluble RANKL and M-CSF, which is sensitive to treatment with RN1734, a TRPV4-specific antagonist (*15*), showing that PGE_2 may activate TRPV4 in a similar way. Acidosis itself could release PGE_2, implying that it would also release arachidonic acid, the precursor of PGE_2 (*33*), and downstream metabolites, which could activate TRPV4 channels. Arachidonic acid and its metabolites can activate a wide variety of cation channels (*34,35*).

Ca^{2+} influx through TRP channels appears to be important to the formation of large osteoclasts, because lowering the extracellular Ca^{2+} concentration using EGTA, a Ca^{2+}-specific chelating reagent, reduced the degree of acidosis-promoted osteoclast formation. ASICs are unlikely important to acidosis-promoted osteoclast formation, because Ca^{2+} influx appears to be of primary importance in acidosis-promoted osteoclastogenesis. A Gs-related system is also unlikely to act on preosteoclasts in acidosis-promoted osteoclastogenesis, because dibutylyl-cAMP, a membrane-permeable derivative of cAMP, and forskolin, an adenylatecyclase activator, inhibit osteoclast formation (*36*).

TRPV4 channels appear to contribute to acidosis-promoted osteoclast formation because RN1734, a TRPV4-specific antagonist, partially inhibited, and 4-α PDD, a TRPV4-specific agonist, enhanced osteoclst formation under mild acidosis. Other unidentified TRP family cation channels permeable to Ca^{2+} may also contribute to acidosis-promoted osteoclast formation. In our experiment Ruthenium red, a general blocker of TRP channels, potently suppressed the osteoclastogenesis. Ruthenium red also blocks Ca^{2+}-dependent Ca^{2+} release (CICR) *via* ryanodine receptors. Another CICR blocker, dantrolen, did not show inhibitory effects on acidosis-promoted osteoclast formation, suggesting that Ruthenium red primarily acted on TRP channels in this system (*15*).

TRPV2 channels, members of the TRPV family, are sensitive to temperature and mechanical force, and necessary for osteoclast formation. These channels might also be candidates for the acidosis-sensitive machinery which drives osteoclast formation, partly because TRPV2 has structural homology with TRPV1 and TRPV4, is induced by RANKL, is responsible for Ca^{2+} oscillation during preosteoclast differentiation, and is maintained until a comparatively late phase of osteoclast formation (*37*). Studies using specific agonists and antagonists should provide clues as to whether this is the case or not. Anti-OGR1 specific to the extracellular domain of OGR1 was not able to suppress large osteoclast formation when added at the last phase of acidosis-promoted osteoclast formation.

6. Acid-sensing machinery

Osteoclasts are vulnerable to apoptosis under physiological conditions. Acidosis is reported to promote survival through NFATc1-independent and PKC-dependent pathways under the control of OGR1 (*38*). Acidosis is also reported to potentiate osteoclast survival to activate bone resorption *via* upregulation of osteopontin, promoting cell survival through integrin binding, augmentation of adhesion and spreading *via* activation of pyk-2, Cbl-b and src activation (*39*). However, Teramoto has reported that acidosis does not modulate osteoclast survival (*14)*.

7. Acidosis and *RANKL* gene expression

Several groups have addressed how acidosis works *via* OGR1. They used osteoblastic cells and calvarial organ cultures. Acidosis activated OGR1 to elevate intracellular Ca^{2+} levels *via* Gq stimulation, resulting in cyclooxygenase 2 (COX-2) expression. This led to the production of PGE_2, which is reported to activate osteoblasts to induce RANKL expression. siRNA against OGR1 blocked acidosis-induced COX-2 expression in a human osteoblastic cell line. YM-254890, a Gq antagonist, and PKC inhibitors blocked COX-2 expression at low pH (*40*). This result is in line with a report that acid-induced PGE_2 is essential for Ca^{2+} release from cultured calvariae. Pharmacological blocking of intracellular Ca^{2+} was able to suppress COX-2 expression, PGE_2 production and RANKL expression (*41*). This cascade from OGR1 to COX-2 and RANKL might be an event in the induction process by acidosis. Acidosis-promoted osteoclast formation in our experiments, where bone marrow cells were supported by soluble RANKL and M-CSF, was not blocked in the presence of Indomethacin, a general

inhibitor of cyclooxygenases, implying that PGE_2 production does not have primary role in the late phase of osteoclast formation. We have observed that the role of COX-2 in RANKL induction appears to be during a limited period in the course of osteoclast formation (unpublished data).

8. Bicarbonate ions repress osteoclast formation

As mentioned at the beginning, alkalosis suppresses bone resorption. One reason for this is that bicarbonate ions are able to activate a soluble type adenylatecyclase, which produces cAMP (*42*). cAMP-elevating reagents acting on osteoblasts, PGE_2 and PTH (parathyroid hormone), generally induce RANKL expression. On the other hand, those acting on osteoclasts suppress osteoclast formation and activation, for example, forskolin, dibutyl cAMP and calcitonin (*36*). Thus local and systemic control of HCO_3^- ions appears to be of great importance for maintaining bone mineral content.

9. Acidosis and osteoblast-lineage differentiation, survival and functions

Osteoblast differentiation is inhibited under acidosis. In a regular system, where the osteoblasts are derived from bone marrow cells in a 5% CO_2/HCO_3 system, osteoblasts differentiate better at higher than lower pH (Figure 4A). Acidosis is reported to impede the production of collagen type 1, osteocalcin and other osteoblast marker proteins (*43*). Inflammatory cytokines, tumor necrosis factor (TNF)-α and IL-1 β also deteriorate osteoblast formation with different strengths (Figure 4B). TNF-α showed potent inhibitory activity under acidotic conditions. We confirmed that acidosis and TNF-α cooperate to inhibit osteoblast differentiation from bone marrow cells (Figure 4C).

Connexins are a group of proteins, which contain short cytoplasmic N-terminal sequences, intracellular four transmembrane domains and long intracellular C-terminal sequences and assemble as the hexamer connexon on the plasma membrane (*44*). So far the

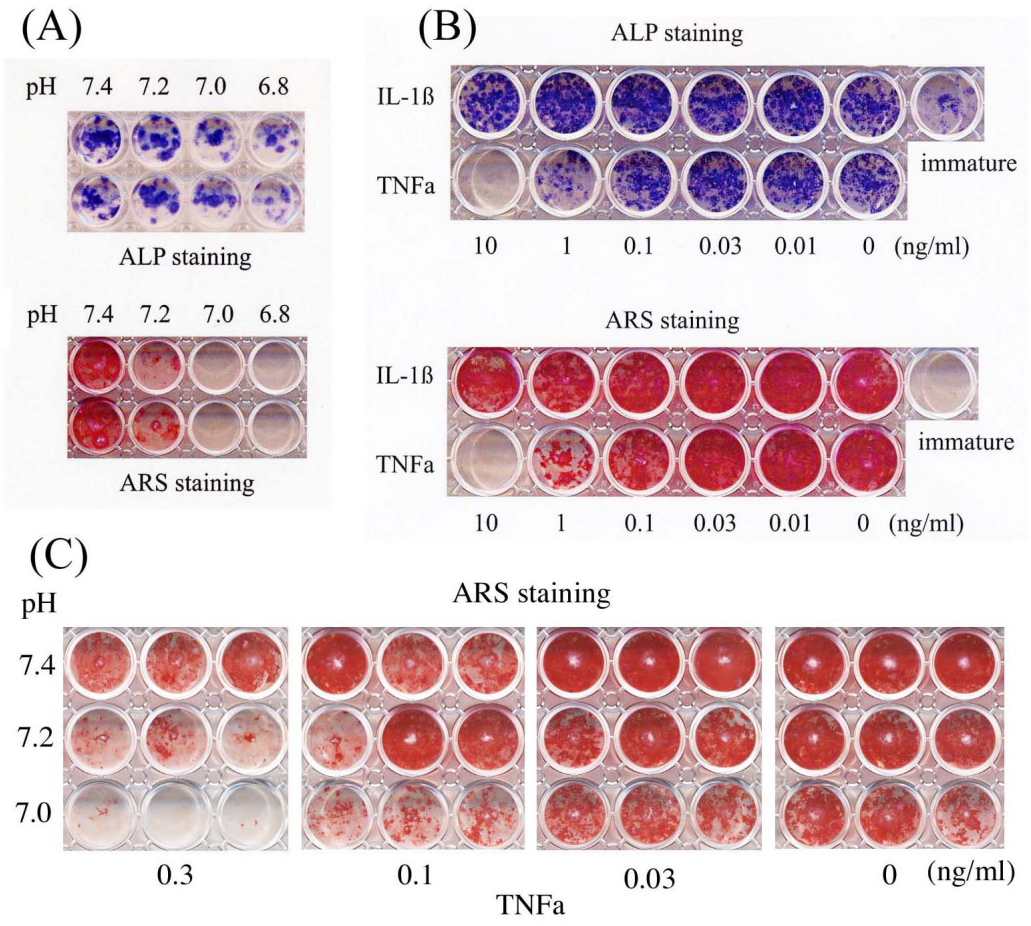

Figure 4. Acidosis impedes the differentiation of osteoblast lineage cells from bone marrow cells. Mouce bone marrow cells (1,000,000 cells/1 mL/well) were cultured in 24-well plates in α-MEM-10% fetal calf serum in the presence of 50 μg/mL ascorbic acid 2-phosphate (Ascp) and 10 mM glycerol 2-phosphate (Gp) at pH 7.4, 7.2, 7.0, and 6.8. The effects of IL-1 beta (IL-1β) and TNF-α were tested at pH 7.3. The culture medium was changed every third day in all cases. Cultures without Ascp and Gp were called immature in the figure. **(A)** Alkaline phosphatase staining (ALP) was conducted on day 10 for early differentiation of osteoblasts to test early differentiation of osteoblasts. **(B)** Alizarin red S staining (ARS) was performed on day 21 to certify nodule calcification. **(C)** TNF-α potently acts against osteoblast differentiation under acidotic conditions.

group has 21 members. Connexin 43 (Cx43) is the most dominant in bone tissue. There have been numerous investigations into the roles of Cx43 using many types of tissues and cells, including cell differentiation and cell survival (*45,46*). Cx43 is found in osteoblast-lineage cells, especially in osteocytes. Cx43 connexons are thought to act as hemichannels, platforms for the assembly of proteins through C-terminal sequences, intercellular channels to transmit or exchange Ca^{2+}, other ions, cAMP and small peptides and nucleotides with a molecular weight of less than around 1,000. There are several reports on Cx43 knockout mice specific to the osteoblast-lineage (*47*). A delay of osteoblast lineage differentiation and deterioration of function and survival are common phenotypes of Cx43 conditional knockout mice (*48,49*), although the mechanism remains to be elucidated. Astrocytes are reported to internalize Cx43 under acidosis (*50*). This is an interesting example because osteoblast differentiation is delayed when Cx43 is depleted (*51*). The survival of osteocytes is influenced by Cx43 (*48*). These examples suggest that the differentiation of osteoblast-lineage cells from mesenchymal stem cells and their survival would be impeded under acidotic environments. In addition to acidosis, hypoxic conditions cause Cx43 internalization (*52*).

10. Acidosis, hypoxia and reactive oxygen species

Hypoxic conditions impair bone formation, partly because oxygen is necessary for the production of collagen molecules, rich in hydroxylized lysine and proline residues. Biosynthesis generally requires energy for putting materials into whole molecules that hold ordered structures. When tissues are left in a hypoxic environment, aerobic metabolic cascades slow down and an anaerobic respiratory system dominates, producing more lactic acid. Degenerated biomaterials often contain nonvolatile acidic products, sulfates, phosphates and so on. Thus, acidosis is closely related with hypoxia and both are favorable for bone resorption (*4,53*). Both would work cooperatively, impeding bone formation and accelerating bone resorption.

Acidosis is an environmental factor for deteriorating bone formation, promoting osteoclast formation and activity, impeding osteoblast differentiation. Although how acidosis acts on preosteoclasts and preosteoblasts to exert inverse effects, promotion and suppression of differentiation, respectively, remains to be elucidated, several hints appear to be in the researches regarding the roles of reactive oxygen species in bone metabolism.

RANKL stimulates osteoclast differentiation by stimulating several signal pathways converging on NFATc1 activation, where reactive oxygen species induced by RANKL stimulation promote long-lasting Ca^{2+} oscillation required for osteoclast formation (*54*). Glucocorticoide and TNF-α are known to suppress osteoblast differentiation, where reactive oxygen works to eventually decrease the amount of active form β-catenin, a key transcriptional factor required for osteoblast differentiation (*55*). In cancer cells acidic environment itself leads to the generation of reactive oxygen species (*56*).

11. Conclusions

Acidosis is deeply involved in a variety of diseases and pathological conditions, promoting bone resorption and impeding bone formation. It therefore could be a candidate for intervention in the treatment of diseases.

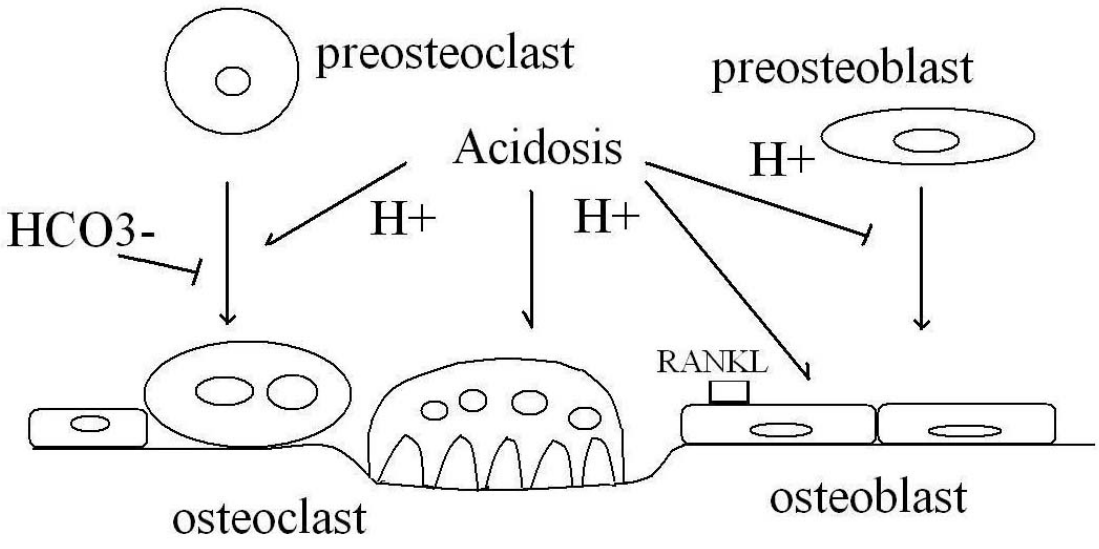

Figure 5. Acidosis promotes osteoclast differentiation and activation and inhibits osteoblast differentiation. This is a schematic indication of the contens of this review. Acidosis has promotive effects on osteoclast lineage cells. On the other hand acidosis acts against osteoblast differentiation.

The acid-base balance is a basic factor for the body. Therefore, this influences a wide variety of targets, conferring accents on more system-specific reactions. Elucidation of the relationship among acidosis, hypoxia, redox state, RANKL production, mechanical force, Wnt signaling and inflammatory cytokines would provide a more precise understanding of bone physiology and pathology of bone-related diseases. Figure 5 is a schematic drawing of the contents of the text.

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