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# Osteoclast Precursors Display Dynamic Metabolic Shifts toward Accelerated Glucose Metabolism at an Early Stage of RANKL-Stimulated Osteoclast Differentiation

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**Key Words**

Bone • Osteoclastogenesis • Metabolic diseases • Osteoporosis • Metabolic substrate

**Abstract**

Mature osteoclasts have an increased citric acid cycle and mitochondrial respiration to generate high ATP production and ultimately lead to bone resorption. However, changes in metabolic pathways during osteoclast differentiation have not been fully illustrated. We report that glycolysis and oxidative phosphorylation characterized by glucose and oxygen consumption as well as lactate production were increased during receptor activator of nuclear factor-κB ligand (RANKL)-induced osteoclastogenesis from RAW264.7 and bone marrow-derived macrophage cells. Cell proliferation and differentiation varied according to glucose concentrations (0 to 100 mM). Maximal cell growth occurred at 20 mM glucose concentration and differentiation occurred at 5 mM concentration. Despite the similar growth rates exhibited when cultured cells were exposed to either 5 mM or 40 mM glucose, their differentiation was markedly decreased in high glucose concentrations. This finding suggests the possibility that osteoclastogenesis could be regulated by changes in metabolic substrate concentrations. To further address the effect of metabolic shift on osteoclastogenesis, we exposed cultured cells to pyruvate, which is capable of promoting mitochondrial respiration. Treatment of pyruvate synergistically increased osteoclastogenesis through the activation of RANKL-stimulated signals (ERK and JNK). We also found that osteoclastogenesis was retarded by blocking ATP production with either the inhibitors of mitochondrial complexes, such as rotenone and antimycin A, or the inhibitor of ATP synthase, oligomycin. Taken together, these results indicate that glucose metabolism during osteoclast differentiation is accelerated and that a metabolic shift towards mitochondrial respiration allows high ATP production and induces enhanced osteoclast differentiation.



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## Introduction

Cellular metabolic pathways are strictly regulated and provide flexible modulation of cellular responses to environmental changes, nutrition, growth factors, and aging. The aging process in the human body induces changes in metabolism and decreases bone quality [1, 2]. Aging-related disorders, such as tumors and diabetes, result in metabolic abnormalities with alterations in glucose utilization. Tumors have a shift in their metabolism from oxidative phosphorylation to glycolysis, which results in elevated glycolysis even in the presence of oxygen. Several tumors with high glucose-utilizing metabolisms via glycolysis lead to bone diseases [3-6]. Type I diabetes, a state which is characterized by hyperglycemia and no insulin secretion, is associated with bone loss through its ability to decrease bone remodeling, or osteoporosis [7].

Bone is the only solid tissue in the human body. As a living and dynamic tissue, bone is remodeled by a delicate balance between bone-forming osteoblasts and bone-resorbing osteoclasts. Recently, shifts in metabolic pathways have been observed during osteoblast differentiation [8]. When osteoblasts were differentiated from ascorbic acid treated calvaria-originated cells, cellular metabolic activity, glycolysis, and respiration fluctuated during sequential differentiation stages [8]. In the undifferentiated period, the ratio of lactate production to glucose consumption was approximately 2, indicating that the major energy source in this period depends on glycolysis. Furthermore, the rate of cellular respiration at the early stages of differentiation was markedly increased to allow active proliferation. In contrast, both glycolysis and respiration were enhanced during middle and late stages of osteoblast maturation in order to biosynthesize the components demanded for differentiation.

Another study reported that high metabolic activity and ATP production could be required for the serial processes of osteoclast differentiation, including proliferation, migration, and fusion to form multinuclear cells, as well as for bone resorption in mature osteoclasts. This possibility is consistent with the following evidence: (i) exogenous ATP stimulated osteoclast formation and resorption pit formation [9], (ii) that osteoclasts have abundant mitochondria [10], (iii) that metabolic enzymes involved in the citric acid cycle and oxidative phosphorylation were upregulated during osteoclast differentiation [11], and (iv) that a large amount of ATP is used by vacuolar-type $H^+$-ATPases in mature osteoclasts to pump protons into an extracellular resorption area [10]. These events lead to an acidification that dissolves apatite crystal and degrades organic bone matrix.

Although metabolic changes in bone-relating cells appear to be implicated in bone remodeling, little is known about endogenous metabolic regulation at the early stages of osteoclast differentiation. The goal of our study was to examine how metabolism is regulated during osteoclast differentiation and whether metabolic substrates and disturbances can modulate osteoclast differentiation. Our findings demonstrate that glucose metabolism, such as glycolysis and mitochondrial respiration, is accelerated at an early stage of the receptor activator of nuclear factor-κB ligand (RANKL)-induced osteoclast differentiation. In addition, we found that metabolic alterations could modify osteoclast differentiation.

## Materials and Methods

*Cell culture*

The murine monocytic RAW264.7 cell line is capable of differentiating into osteoclasts by stimulation with RANKL. We maintained this cell line under a humidified atmosphere of 5% $CO_2$ at 37°C in Dulbecco's Modified Eagle's Medium (DMEM) containing 25 mM glucose (Invitrogen, cat. no. 11971, Carlsbad, CA), 10% fetal bovine serum (FBS), and antibiotics.

*Osteoclast differentiation*

Bone marrow-derived monocytes were isolated from the tibia and femur of 6-week-old C57BL/6 female mice by flushing the bone marrow cavity with minimum essential medium-alpha (α-MEM) containing 5.5 mM glucose and 1 mM pyruvate (Invitrogen, cat. no. 12571). The cells were centrifuged and the red blood cells were removed by a treatment with ACK buffer (0.15 mM $NH_4Cl$, 1 mM $KCO_3$, and 0.1 mM EDTA, pH 7.4) for 30 sec. The remaining cells were then washed with α-MEM. Next, the harvested cells were cultured with α-MEM containing 10% FBS and 5 ng/ml macrophage colony-stimulating factor (M-CSF, Genetics Institute, Cambridge, MA) for 12 h. The non-adherent cells were collected, seeded on a 24-well culture plate at $5 \times 10^4$ cells /well, and incubated for 3 days with α-MEM containing 30 ng/ml M-CSF to generate bone marrow-derived macrophages (BMMϕ) into osteoclast precursors. In our study, glucose and pyruvate-free DMEM (Invitrogen, cat. no. 11966) was used to assess the effects of metabolic substrates on osteoclast differentiation. We called this medium DMEM-W/O. BMMϕ were adapted in DMEM-W/O with either glucose only (5 mM or 40 mM) or glucose plus pyruvate (1 mM) containing 30 ng/ml M-CSF and 10% FBS that had been dialyzed for 24 h with phosphate-buffered saline (PBS) to remove metabolites, such as glucose and pyruvate. Cells were then cultured with soluble RANKL (100 ng/ml), which is a key factor stimulating the differentiation of osteoclasts [12], for 4 days to generate osteoclasts. The media were changed on day 2.

To induce differentiation of RAW264.7 macrophage-like cells into osteoclasts, the cells were cultured in the presence of

100 ng/ml RANKL for 4 days. In addition, to test the effects of metabolic substrates on differentiation, cells at a concentration of 1.5 x $10^4$ cells/1 ml were seeded on 24-well culture plates and maintained for 24 h in culture media containing 10% dialyzed FBS and glucose ranging in concentrations from 0 mM to 100 mM, glucose (5 mM or 40 mM), or glucose plus pyruvate (5 mM). Afterwards, cells were cultured with RANKL for 4 days. Fresh media were administered on day 2.

*TRAP staining and activity*

Cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP) and tartrate-resistant acid phosphatase-positive multinucleated (> 3 nuclei) cells (TRAP(+) MNCs). Positive staining cells were counted as osteoclasts under a light microscope. The specific TRAP activity was determined as described elsewhere by measuring the absorbance of the stained cells at 405 nm [13].

*MTT assay*

RAW264.7 cells were plated at a density of 1 x $10^3$ cells/well/100 µl on 96-well culture plates at various medium conditions, including broad range of glucose concentrations from 0 mM to 100 mM and the combination of glucose and pyruvate. The cells were also incubated for 30 min in a colorimetric substrate MTT (3-(4,5-dimethylthiazol-2-thiazolyl)-2,5-diphenyltetrazolium bromide; Sigma, St. Louis, MO) at a final concentration of 500 µg/ml. After washing with PBS, the cells were dissolved in dimethyl sulfoxide and the cell viability was examined by reading the absorbance of each sample at 570 nm.

*Measurements of glucose and lactate contents in culture media*

RAW264.7 cells and BMMϕ were cultured in various conditions for the indicated times. Glucose concentrations in the conditioned medium were determined by using a Glucose (GO) Assay Kit (Sigma). To measure lactate contents in the conditioned medium, the medium was heat-inactivated at 80°C for 10 min and centrifuged at 12,000 x g for 10 min at 4°C. A 500 µl reaction mixture containing 0.32 mM glycine (pH 9.2), 0.32 mM hydrazine, 9.6 mM nicotinamide adenine dinucleotide, and 3 U/ml lactate dehydrogenase (Sigma) was incubated at room temperature for 2 min, after which the reaction was started by adding 500 µl of the prepared medium. Lactate concentration was measured by reading the absorbance increase at 340 nm using a DU® 650 spectrophotometer (Beckman Coulter, Fullerton, CA). This reading reflected the reduction of nicotinamide adenine dinucleotide in the sample.

*Oxygen consumption*

RAW264.7 cells and BMMϕ (5 x $10^4$ cells/well/200 µl) were seeded into a 96-well plate with the BD™ Oxygen Biosensor System (BD Biosciences, Franklin Lakes, NJ). After plating, cells were adapted for 12 h and cultured in various conditioned media. The amount of fluorescence was analyzed at the indicated times by a Fluostar Optima (BMG Labtechnologies, Durham, NC) with an excitation at 485 nm and an emission at 590 nm.

*Measurement of intracellular peroxides and free thiol groups*

Intracellular peroxides and free thiol groups were analyzed with 2´,7´-dichlorofluorescin diacetate (Sigma) and 5,5´-dithiobis-(2-nitrobenzoic acid) (Sigma), as described elsewhere [14].

*Western blot analysis*

RAW264.7 cells (2 x $10^5$ cells /60-mm dish) were cultured with glucose alone (5 mM or 40 mM) or with the combination of glucose and pyruvate (5 mM) for 24 h. After stimulation with 100 ng/ml RANKL for 10 min, whole cell lysates were prepared with a lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.1% SDS, 2 mM $Na_3VO_4$, 1 mM glycerol phosphate, and 1X protease inhibitor cocktail). The resulting supernatant was collected by centrifugation and fractionated by electrophoresis on a 10% SDS-polyacrylamide gel. The separated proteins were transferred onto an Immobilon-P membrane (Millipore, Bedford, MA) and probed with rabbit antibodies against mitogen-activated protein kinases (MAPKs), extracellular signal-regulated kinase (ERK), phospho-ERK, c-Jun $NH_2$-terminal kinase (JNK), phospho-JNK, p38, and phospho-p38 (Cell Signaling, Beverly, MA). All blots were then probed with a rabbit HRP-conjugated secondary antibody (Cell Signaling) and developed by ECL (Lab Frontier, Korea). The relative phosphorylated protein levels were determined as follows. First, the densitometric intensities of the phosphorylated target protein or its total counterpart from the western blots were measured with a LAS-1000 Plus (Fuji Photo film, Japan). The ratio of the target protein to the total protein levels in each sample was then calculated to correct for differences in protein loading. To obtain the relative levels of protein, this ratio was normalized to the phosphorylated target protein divided by the total counterpart negative control ratio.

*Statistical analysis*

Data were evaluated by analysis of variance using the STATISTICA 6.0 software package. When significant differences were found, pair wise comparisons were performed using Scheffe's adjustment. Differences were considered statistically significant if their associated p-values were less than 0.05.

## Results

### *Metabolic switch to accelerated glycolysis and mitochondrial respiration during RANKL-stimulated osteoclast differentiation*

The main energy source for cell function, proliferation, and differentiation is ATP. ATP is generated by metabolic pathways, such as glycolysis in the cytosol and the citric acid cycle and oxidative phosphorylation in the mitochondria. A large ATP content is required for the serial stages of osteoclast differentiation, cell proliferation,

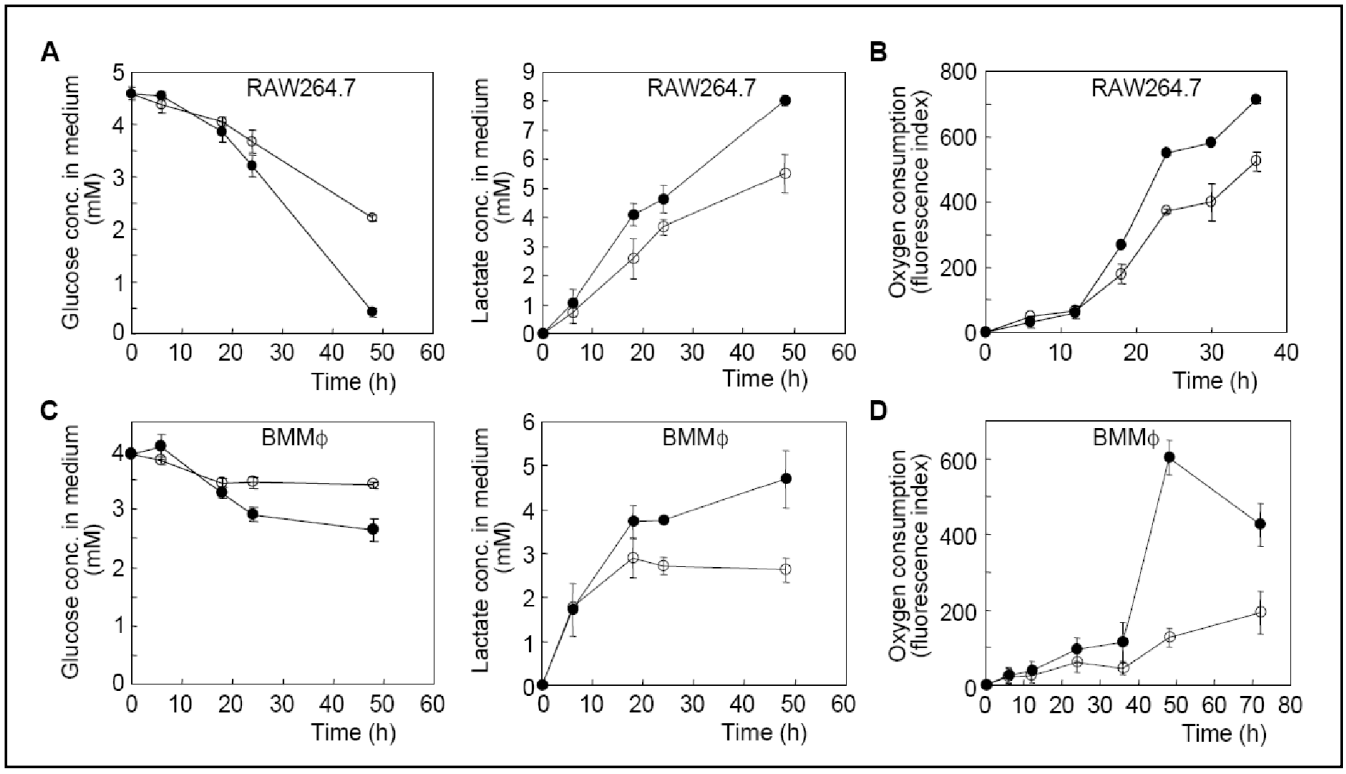


**Fig. 1.** Metabolic changes during RANKL-stimulated osteoclast differentiation. A and C: Glucose consumption and lactate accumulation in culture media. RAW264.7 cells (2 x $10^5$ cells/60-mm dish) and BMMϕ (0.5 x $10^6$ cells/60-mm dish) were cultured in DMEM-W/O containing 10% dialyzed FBS and 5 mM glucose and in α-MEM containing 10% dialyzed FBS and 30 ng/ml M-CSF in the absence or presence of RANKL, respectively. Glucose and lactate contents were assayed in the medium at the indicated times. Data are expressed as the mean ± S.D. (n = 3) from a representative experiment of three independent experiments. B and D: Oxygen consumption. RAW264.7 cells and BMMϕ were seeded into 96-well Oxygen Biosensor plates, cultured, and fluorescence intensity was measured at the indicated times. Assay conditions were the same as in (A) and (C). Data are expressed as the mean ± S.D. (n = 4). o, Control; ●, RANKL.

migration, and the final fusion of mononucleated cells to form multinucleated cells. To test whether metabolic pathways could be altered during RANKL-stimulated osteoclast differentiation, we assessed cellular glycolytic activity by measuring glucose consumption and lactate accumulation in cell culture media. We also determined cellular oxygen consumption in order to analyze mitochondrial oxidative phosphorylation. After RANKL treatment, RAW264.7 cells (Fig. 1A) and BMMϕ (Fig. 1C) both had gradual, time-dependent increases in glucose consumption and lactate accumulation. Moreover, RANKL-stimulated cells had accelerated mitochondrial respiration compared with RANKL-untreated control cells (Fig. 1B, D). Our experimental system has no influence on cell growth for the indicated times (data not shown), indicating that metabolic shift is not due to the effect of cell damage.

Reactive oxygen species are caused by electron leakage via mitochondrial electron transport [15, 16], and intracellular and extracellular acidosis are mainly originated in lactic acid generated from glycolysis [17]. To confirm high cellular metabolic activity, we determined the cellular redox status and pH alterations in the culture media. RAW264.7 cells were cultured in the absence or presence of RANKL for 12 h to measure intracellular peroxides and for 48 h to analyze total thiol groups. Treatment of RANKL significantly increased the level of intracellular peroxides (Fig. 2A, left panel) and subsequent thiol oxidation. These events eventually resulted in a decreased cytosolic thiol content (Fig. 2A, right panel). When extracellular pH changes in the culture media were monitored directly by a pH meter after transfer of the cells to a $HCO_3^-$-free incubator, the extracellular pH of RANKL-treated cells was significantly

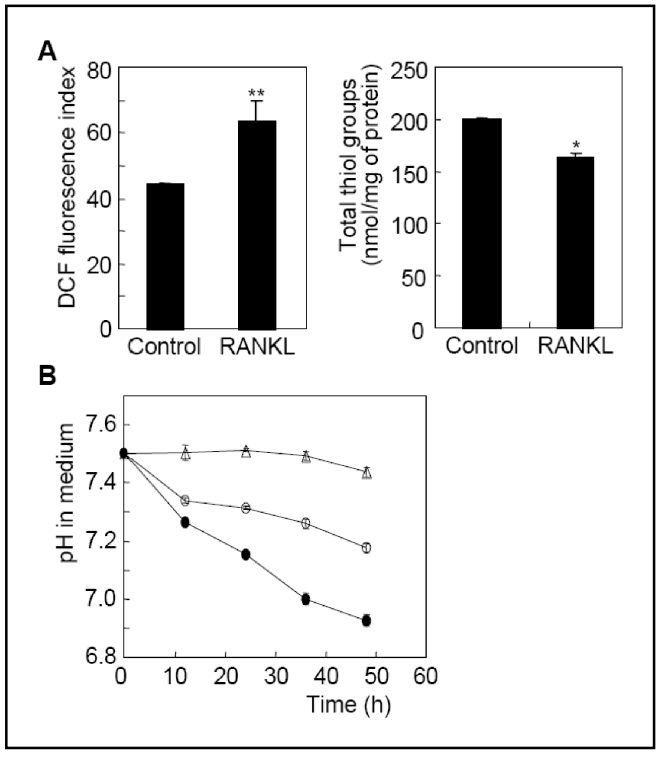


**Fig. 2.** Physiological changes in response to RANKL. A: Determination of the intracellular redox status. RAW264.7 cells were cultured in the absence or presence of RANKL for 12 h to measure intracellular peroxides (left panel) and for 48 h to analyze total thiol groups (right panel) as described in the Figure 1A legend. The contents of intracellular peroxides and total thiol groups were determined in the cytosolic fractions. **P* < 0.01 versus RANKL-untreated control. ***P* < 0.05 versus RANKL-untreated control. B: pH changes in the culture media. RAW264.7 cells (1x10$^6$ cells /60-mm dish/4 ml) were adapted under an $HCO_3^-$-supplied incubator for 6 h and washed with PBS. Cells were then cultured in 1 mM HEPES-buffered DMEM [Since Invitrogen company had no the conditioned medium without sodium bicarbonate, glucose, and pyruvate, we exceptionally used DMEM (Invitrogen cat. no. 31600) without sodium bicarbonate and with 5.5 mM glucose and 1 mM pyruvate to test pH alteration within a narrow range.] and adjusted to a pH 7.5 with NaOH in the absence or presence of RANKL. After transferring to $HCO_3^-$-free incubator, the pH of the culture media was directly measured using a pH meter (Thermo Orion Model 420) at the indicated times. Data are expressed as the mean ± S.D. (n = 3) from a representative experiment of three independent determinations. Δ, medium alone; O, control; ●, RANKL.

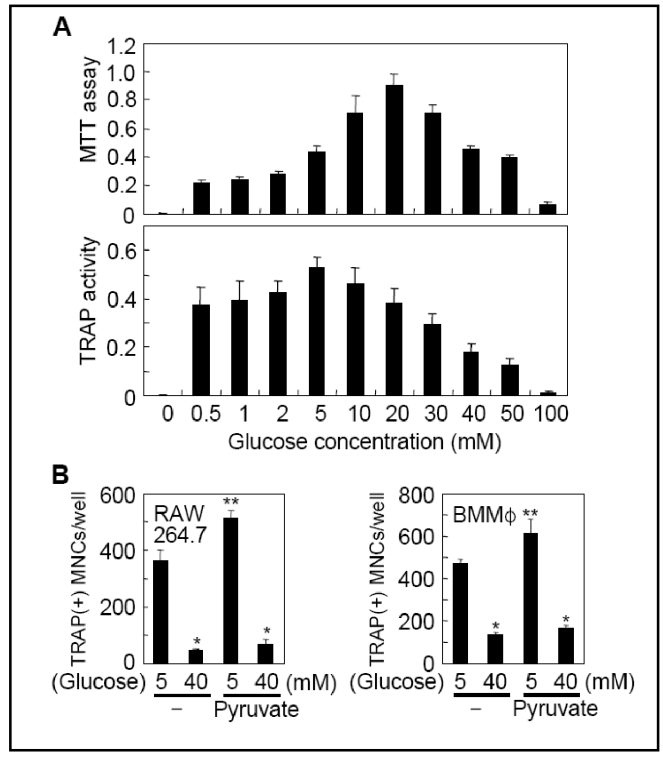


**Fig. 3.** Effect of metabolic substrates on osteoclast differentiation. A: Effect of various glucose concentrations on cell growth and differentiation. RAW264.7 cells (1 x 10$^3$ cells/well) were seeded into a 96-well culture plate and cultured in DMEM-W/O with various concentrations of glucose in the absence (upper panel) or presence (lower panel) of RANKL. On day 5, the extent of cell growth and differentiation was determined by a colorimetric MTT assay (upper panel) and TRAP activity (lower panel), respectively. Data are expressed as the mean ± S.D. (n = 4). B: Synergistic effect of pyruvate on osteoclast differentiation. RAW264.7 cells and BMMϕ were seeded and then cultured in DMEM-W/O with glucose only (5 mM or 40 mM) or glucose plus pyruvate (5 mM in RAW264.7 cells; 1 mM in BMMϕ) as described in the "Materials and Methods" section. After incubation with RANKL (RAW264.7 cells) or M-CSF plus RANKL (osteoclast precursors) for 4 days, the number of TRAP(+) MNCs was counted under a light microscope. Data are expressed as the mean ± S.D. (n = 3). **P* < 0.01 versus 5 mM glucose-treated cells. ***P* < 0.05 versus 5 mM glucose-treated cells.

lower than the extracellular pH of RANKL-untreated control cells. This decrease in extracellular pH occurred in a time-dependent manner (Fig. 2B). Our results indicate that immature osteoclasts display high rates of glycolysis and mitochondrial respiration in an early stage of RANKL-stimulated osteoclastogenesis.

### *Regulation of RANKL-stimulated osteoclast differentiation by metabolites*

Progressive osteoblast differentiation coincides with changes in cellular metabolism and mitochondrial activity. Thus, these changes likely play a key role in osteoblast function [8]. Our study shows that immature osteoclasts

display high rates of glycolysis and mitochondrial respiration. To test whether metabolites could regulate RANKL-stimulated osteoclast differentiation, we studied the effects of various glucose concentrations and the combination of glucose and pyruvate on osteoclastogenesis. Cell growth increased in a dose-dependent manner for glucose concentrations up to 20 mM. However, cells treated with glucose concentrations over 20 mM had a sequential decrease in cell growth (Fig. 3A, upper panel). TRAP activity, which is a hallmark of osteoclastogenesis, was also maximally increased when cells were treated with 5 mM of glucose. This TRAP activity gradually decreased with glucose concentrations over 5 mM (Fig. 3A, lower panel). While similar growth patterns were observed in cells treated with either 5 mM or 40 mM glucose, the extent of osteoclast differentiation with the 40 mM glucose treatment was significantly lower than the amount of differentiation observed with the 5 mM glucose treatment. We also confirmed that the growth of cells treated with 5 mM glucose is analogous to that of cells treated with 40 mM glucose, determining via the trypan blue method (data not shown). As shown in Figure 1 and Figure 3A, the data suggest a possibility that RANKL-stimulated osteoclastogenesis could be regulated by metabolic shifts. To manipulate cellular metabolism, cultured cells were treated with pyruvate, a compound which accelerates mitochondrial respiration (Fig. 4) [18]. The number of TRAP(+) MNCs generated from RAW264.7 cells and BMMϕ in the presence of RANKL increased significantly more when cells were cultured in 5 mM glucose than when the cells were cultured in 40 mM glucose. When the cells were exposed to a combination of glucose and pyruvate, the number of TRAP(+) MNCs was synergistically increased compared to the number of cells generated by treatments with glucose alone (Fig. 3B). To examine whether RANKL-stimulated osteoclast differentiation could be controlled by osmotic stress, we counted the number of TRAP(+) MNCs after treating RAW264.7 cells for 4 days with either 40 mM glucose consisting of 5 mM D-glucose and 35 mM L-glucose, 5 mM D-glucose, or 40 mM D-glucose in the presence of RANKL. The number of TRAP(+) MNCs generated under conditions of 40 mM glucose containing both 5 mM D-glucose and 35 mM L-glucose was similar to the number of TRAP(+) MNCs generated with 5 mM D-glucose. However, the number of cells generated under both these conditions was significantly higher than when cells were cultured with 40 mM D-glucose (data not shown), suggesting that osmotic stress has no influence on osteoclast differentiation.

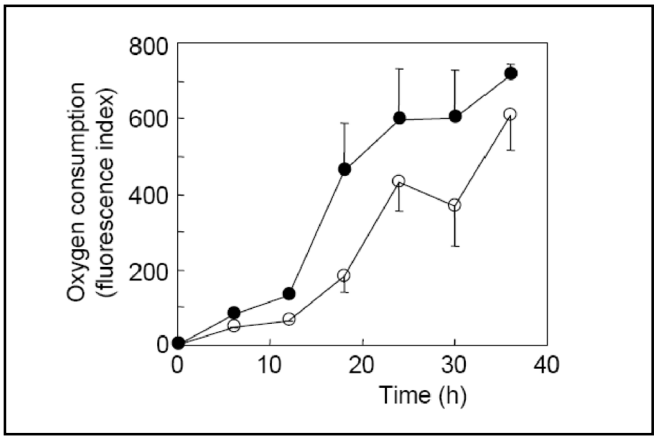


**Fig. 4.** Changes in oxygen consumption by pyruvate. RAW264.7 cells (5 x $10^4$ cells/well) were seeded into 96-well Oxygen Biosensor plates, adapted for 12 h, and cultured further in DMEM-W/O with either glucose alone (5 mM) or glucose (5 mM) plus pyruvate (5 mM) in the absence of RANKL. Fluorescence intensity was measured at the indicated times. ○, glucose alone; ●, glucose plus pyruvate.

To confirm whether the addition of both glucose and pyruvate to the culture medium can lead to an altered metabolism, we analyzed metabolic shifts. At 5 mM glucose concentrations, the glucose metabolism (glucose consumption and lactate accumulation in culture media) in both RANKL-treated and -untreated cells was diminished by the addition of pyruvate to the cultures (Fig. 5A). Similarly, extracellular pH decreased in RANKL-treated cells at both the 5.5 mM and the 40 mM glucose concentrations, while the addition of pyruvate significantly induced the increase of the extracellular pH at both glucose concentrations in RANKL-treated and -untreated cells (Fig. 5B). These results are consistent with an increase in both glucose consumption and lactate accumulation in culture media that can be attributed to the addition of pyruvate to the cultures. Although glucose consumption at 40 mM glucose concentration levels could not be distinguished between RANKL-treated and -untreated cells, lactate accumulation and pH alterations in the culture media had similar patterns in cells treated with 5 mM glucose (Fig. 5A , B). In addition, after a 30 h incubation with pyruvate, cellular oxygen consumption increased in a time-dependent manner (Fig. 5C and

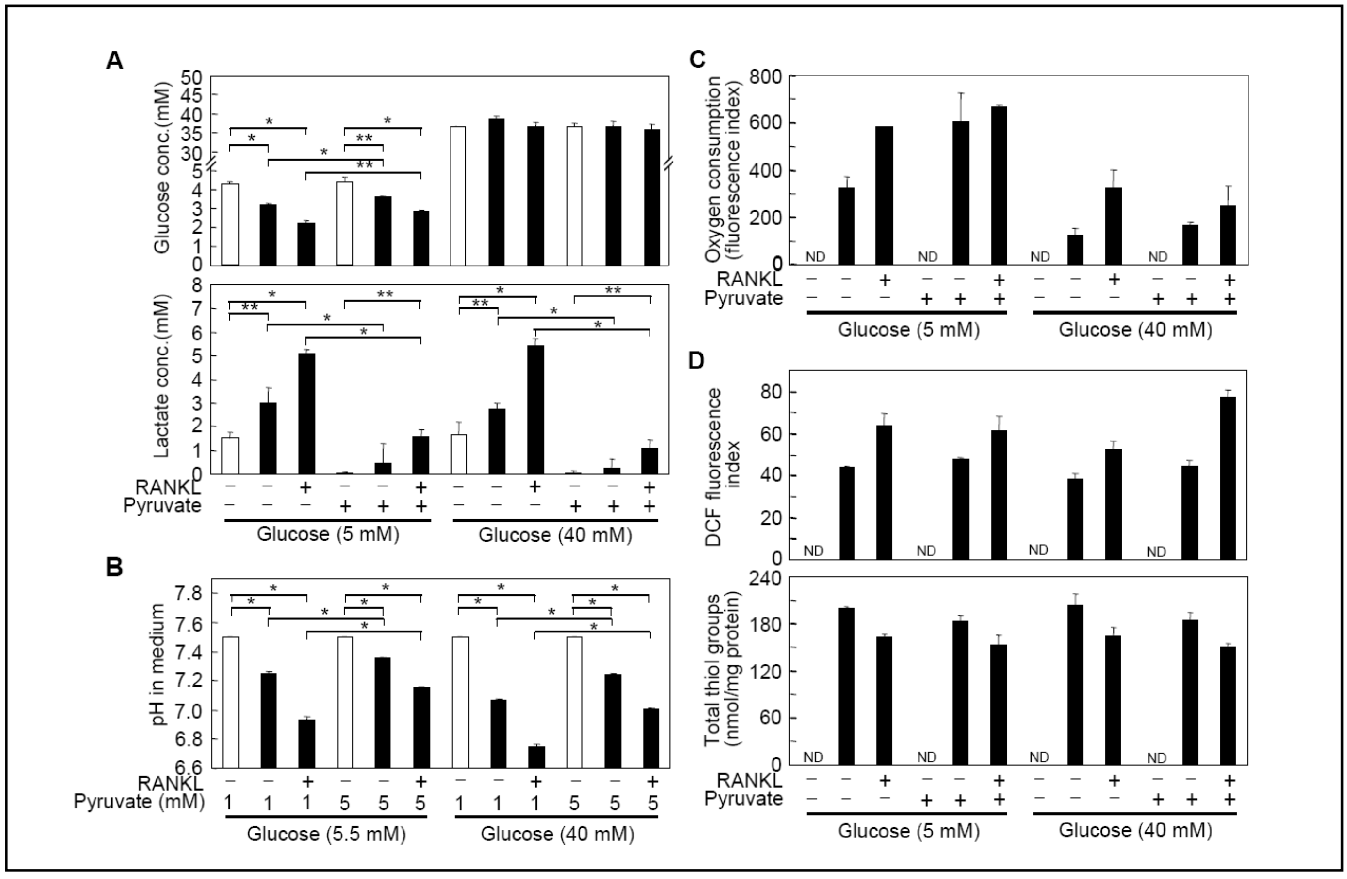


**Fig. 5.** Physiological changes in response to metabolic substrates. A: Glucose consumption and lactate accumulation. RAW264.7 cells were seeded as described in the Figure 1A legend and adapted in DMEM-W/O containing either glucose alone (5 mM or 40 mM) or the combination of pyruvate (5 mM) for 24 h. Cells were further cultured in the absence or presence of RANKL for 24 h. Glucose consumption (upper panel) and lactate accumulation (lower panel) were assessed in the cell culture medium. Data are expressed as the mean ± S.D. (n = 3). *$P < 0.01$. **$P < 0.05$. B: pH changes in the cell culture medium. Cells were adapted to the culture medium for 6 h before a 24 h incubation in 1 mM HEPES-buffered DMEM (pH 7.5) with either 0 and 34.5 mM glucose (final concentrations equal to 5.5 and 40 mM glucose) or 0 and 4 mM pyruvate (final concentrations equal to 1 and 5 mM pyruvate) under an $HCO_3^-$-free incubator. The pH in the culture medium was monitored with a pH meter 24 h after RANKL stimulation. A white bar (□) indicates medium alone. Data are expressed as the mean ± S.D. (n = 3). ND; not determined. *$P < 0.01$. C: Oxygen consumption. RAW264.7 cells (5 x $10^4$ cells/well) were seeded into a 96-well Oxygen Biosensor plate. Oxygen consumption was measured 30 h after the initial culture as in A. D: determination of the intracellular redox status. The contents of the intracellular peroxides and total free thiol groups were assessed as in Figure 2A. ND: not determined.

Fig. 4). This result is consistent with previous observations [18]. Moreover, we observed that oxygen consumption was decreased in cells treated with 40 mM glucose compared to cells treated with 5 mM glucose (Fig. 5C). These results indicate that high glucose levels suppress mitochondrial respiration and that pyruvate treatment leads to metabolic shifts in glycolysis toward oxidative phosphorylation. However, cellular redox status was not significantly different in our model system (Fig. 5D). These cumulative data suggest the possibility that metabolic shifts can regulate osteoclastogenesis.

*Metabolic shifts regulate RANKL-induced osteoclast differentiation signaling events*

Elevated extracellular glucose levels activate p38 MAPK in osteoblasts and endothelial cells [19, 20], but fail to change ERK and JNK activities. Additionally, pyruvate stimulates JNK1 activity but not JNK2 and p38 activities; however, in HeLa cells, pyruvate induces a small increase in ERK activity [21]. Because the activation of metabolite-induced signaling is cell type dependent, we determined whether metabolic shifts in RAW264.7 cells could regulate RANKL-stimulated signals, including the

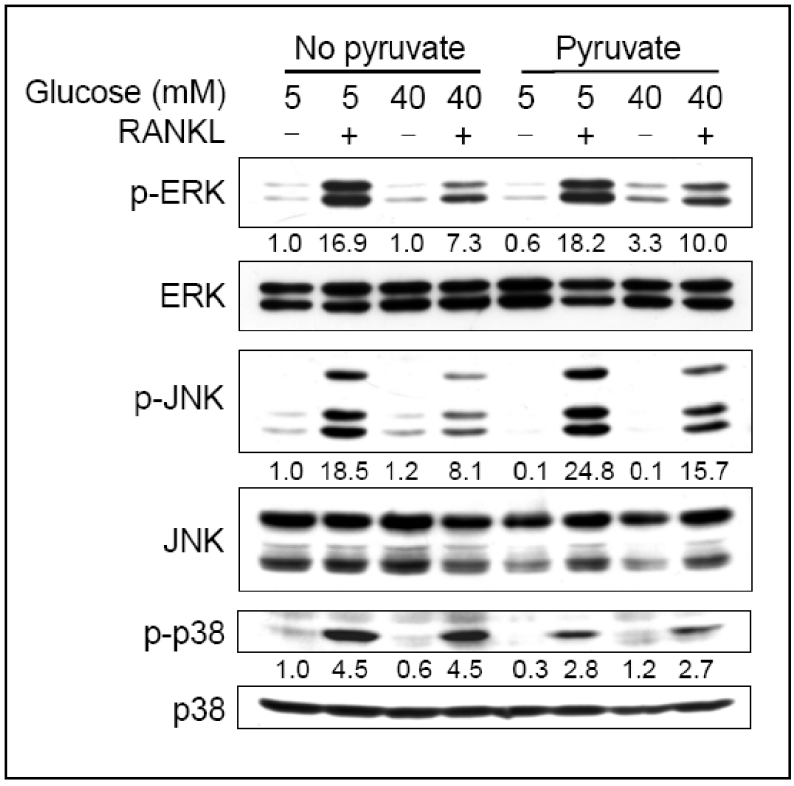


**Fig. 6.** Effect of a metabolic substrate on RANKL-stimulated signaling. RANKL-stimulated signaling. After RAW264.7 cells (2 x $10^5$ cells /60-mm dish) were adapted for 24 h in DMEM-W/O with glucose (5 mM or 40 mM) and the combination of glucose and pyruvate (5 mM), the cells were stimulated with RANKL for 10 min. Whole-cell lysates were generated and immunoblotted with specific antibodies to p-ERK, ERK, p-JNK, JNK, p-p38, and p38. Data are representative of at least three independent experiments.

signals from MAPKs (ERK, JNK, and p38) that are necessary for osteoclastogenesis. The phosphorylation of ERK and JNK was notably enhanced by RANKL treatment at 5 mM compared with 40 mM glucose concentrations. In addition, ERK and JNK activation increased further by pyruvate treatment. Although p38 phosphorylation was enhanced by RANKL, this phosphorylation was not affected by either of the different glucose concentrations or the combination of glucose and pyruvate (Fig. 6). Taken together, these results indicate that metabolic shifts are partially correlated to the alteration of RANKL-induced signaling transmission.

*Blocking ATP generation decreases RANKL-stimulated osteoclast differentiation*

Osteoclastic bone resorption requires high ATP contents [10, 22, 23]. Our study shows that glucose metabolism and mitochondrial respiration were enhanced at an early stage of RANKL-stimulated osteoclastogenesis, ultimately leading to accelerated metabolic shifts toward high ATP production. Additionally, metabolic shifts to respiration by pyruvate induced increased osteoclastogenesis. Therefore, we evaluated the effect of ATP modulation by treating RAW264.7 cells with mitochondrial complex inhibitors (complex I, rotenone; complex II, thenoyltrifluoroacetone; complex III, antimycin A) or oligomycin, an inhibitor of $F_0/F_1$-ATPase. This treatment depleted ATP by inhibiting its production. As shown in Figure 7A, treatment of the inhibitors of complexes I and III decreased the number of TRAP(+) MNCs in a dose-dependent manner, but not that of complex II, known as a minor pathway in mitochondrial electron streams. The number of TRAP(+) MNCs generated by oligomycin were notably decreased in a dose-dependent manner (Fig. 7B). These results are consistent with results obtained with inhibitors of the mitochondrial complexes I and III. The inhibitors did not affect cell growth when they were used at the indicated concentrations and times (data not shown), suggesting that the differential osteoclast formation via ATP content alteration is not caused by cellular cytotoxicity. Taken together, these results indicate that blocking ATP generation significantly decreases RANKL-stimulated osteoclast differentiation.

## Discussion

In general, cellular metabolism is very flexible and meets various physiological demands by altering energy and metabolic substrate utilization. Because mature osteoclasts require high ATP demands to support bone

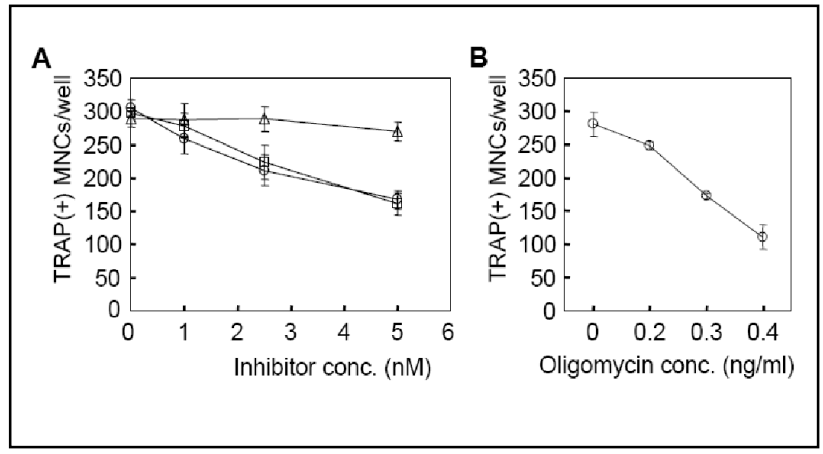


**Fig. 7.** Effect of ATP modulation on osteoclast differentiation. A: Action of mitochondrial complex inhibitors in osteoclast differentiation. RAW264.7 cells (1.5 x $10^4$ cells /well in a 24-well plate) were differentiated into osteoclasts for 4 days in DMEM-W/O containing 5 mM glucose in the presence of mitochondrial complex inhibitors (○, rotenone for complex I; Δ, thenoyltrifluoroacetone for complex II; □ , antimycin A for complex III) and RANKL. After staining with TRAP, the number of TRAP(+) MNCs was assessed as described in the "Materials and Methods" section. Data are expressed as the mean ± S.D. (n = 3). B: Suppressive effect of mitochondrial ATP synthase inhibition on osteoclast differentiation. Cells were cultured for 4 days in DMEM-W/O containing 5 mM glucose in the presence of the mitochondrial ATPase blocker oligomycin and RANKL. Data are expressed as the mean ± S.D. (n = 3).

resorption and biosynthetic intermediates to supply many cellular constituents [10, 22] and because the formation of mature osteoclasts are achieved in at least three stages (including proliferation, migration, and fusion to form multinucleated giant cells), the generation of high ATP levels and other biosynthetic intermediates through metabolic pathways might be required for serial osteoclast differentiation. Here, we observed that metabolic pathways switched to an accelerated glycolytic and oxidative metabolism at an early stage of osteoclastogenesis and that a metabolic shift toward oxidative phosphorylation potentiated RANKL-stimulated osteoclast differentiation.

Osteoclast precursors may undergo differential metabolic shifts at various stages during their differentiation in order to provide the cells with energy and biosynthetic intermediates. Previous report has identified that potential metabolic changes may arise from post-differentiation osteoclastic processes. For example, when comparing mRNA and protein expression profiles occurring during RANKL-stimulated osteoclastogenesis, Czupalla et al. [11] identified upregulated metabolic enzymes related to citric acid cycle, citrate synthase, aconitase, isocitrate dehydrogenase, fumarate hydratase, and malate dehydrogenase, thereby leading to an increased synthesis of ATP. In addition, we here observed that RANKL stimulation induced increased protein levels in the cytosolic fractions (Fig. 8), thus supplying special biosynthetic substrates necessary for the cells to function in a particular proliferation, migration, and fusion stage. Furthermore, glucose consumption and lactate production in RANKL-stimulated cells were higher than that in non-stimulated control cells, representing a high glycolytic rate.

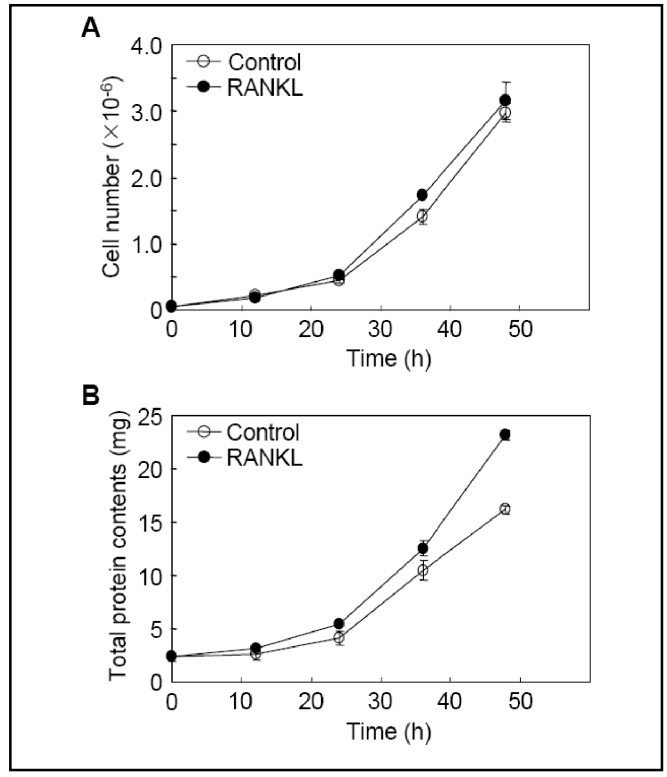


**Fig. 8.** Profiles for cell proliferation and protein content by RANKL stimulation. A: Changes in cell proliferation. RAW264.7 cells (2 x $10^5$ cells/60-mm dish) were cultured in DMEM-W/O containing 5 mM glucose plus or minus RANKL. Cells were harvested by trypsinization and the number of viable cells were counted after trypan blue exclusion at the indicated times. B: Profiles for protein expression. The assay conditions were the same as in A. Cells were resuspended in a lysis buffer (50 mM Tris-HCl, pH 8.0, l mM $MgCl_2$, 4 mM EDTA, and 1 mM DTT), homogenized, and cytosolic fractions were prepared by centrifugation. Protein content was determined using a Bradford reagent (BioRad, Hercules, CA).

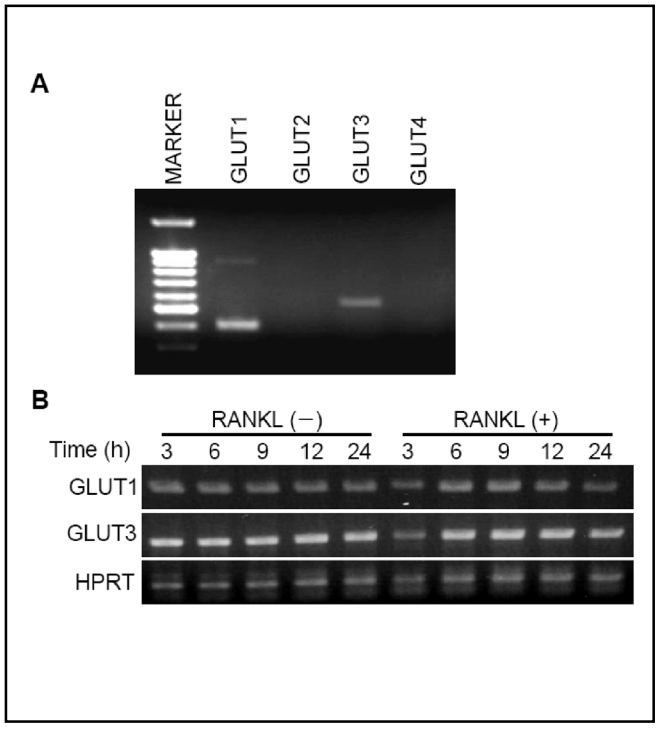


**Fig. 9.** Expression of GLUT in osteoclast precursor RAW264.7 cells in the absence or presence of RANKL. A: Expression pattern for GLUT1 to 4. cDNA was constructed using polymerase chain reaction amplification on total RNA isolated from RAW264.7 cells. Polymerase chain reaction conditions were 30 cycles of the following: denaturation at 94°C for 1 min, annealing at 57°C for 30 sec, and extension at 72°C for 1 min. The specific primers used were as follows: GLUT1, 5′-TGT GTA CTG CGG CCT GAC TAC TG-3′ and 5′-AAC AGC TCC AAG ATG GTG ACC TTC-3′; GLUT2, 5′-TTC GGC TAT GAC ATC GGT GTG-3′ and 5′-AGC TGA GGC CAG CAA TCT GAC-3′; GLUT3, 5′-GTC AAC TTG CTG GCC ATC ATT G-3′ and 5′-TGA GAC AGC TGG AGG ACA ATG G-3′; GLUT4, 5′-AAG ATG GCC ACG GAG AGA G-3′ and 5′-GTG GGT TGT GGC AGT GAG TC-3′; HPRT, 5′-CCT AAG ATG AGC GCA AGT TGA A-3′ and 5′-CCA CAG GAC TAG AAC ACC TGC TAA -3′. The amplified band was fractionated on a 1% agarose gel, stained with ethidium bromide, and visualized with UV illumination. B: Comparison of GLUT1/3 mRNA levels between RANKL-untreated and -treated cells. The expression pattern for GLUT1/3 mRNAs in the the absence or presence of RANKL was analyzed at the indicated times. Co-amplification of the HPRT gene was performed in order to confirm relative expression levels.

Finally, RANKL-treated cells displayed a heightened respiration rate compared with that of untreated control cells. Together, these results demonstrate that a simultaneous activation of glycolytic and oxidative metabolism is necessary for efficient energy and biosynthetic substrate production from glucose. The present findings agree with previous reports that oxidative phosphorylation is increased to support high ATP demands during differentiation of various cell types, including human placental trophoblasts, nerve cells, and human colon adenocarcinoma cells [24-26].

Here, we also showed that the RANKL stimulation that is necessary for inducing osteoclastogenesis was also capable of increasing glucose transport. Others have reported that the entry of glucose into the cell is mediated by the facilitative glucose transporter (GLUT) family [27, 28]. To find which GLUT family is expressed in the osteoclast precursor RAW264.7 cells, we determined the expression of GLUT1, 2, 3 and 4 by a reverse transcription-polymerase chain reaction. We detected GLUT1 and GLUT3, but not GLUT2 and GLUT4 in RAW264.7 cells (Fig. 9A). These results are consistent with the GLUT expression pattern in osteoblasts [29]. Additionally, both GLUT1 and GLUT3 expression was not increased by RANKL stimulation, suggesting that GLUT1/3 levels were constitutively maintained during osteoclastogenesis (Fig. 9B). The efficiency of glucose transport could possibly be regulated by the extent of transcriptional and translational activities of GLUT and by the glucose affinity to GLUT [30]. From these results, an elevation of glucose transport from an extracellular compartment to the cytosolic space by RANKL could be explained by an increased glucose affinity to GLUT1/3.

The mechanism by which metabolites in glucose and pyruvate modulate MAPKs has not been fully elucidated. The RANKL-stimulated ERK and JNK activation in cells treated with a moderate concentration of glucose (5 mM) was reduced in cells treated with a high glucose concentration (40 mM). A previous study reported that elevated glucose activates protein tyrosine phosphatases, thus inactivating MAPKs [31]. Since MAPK activity is reciprocally coordinated by tyrosine phosphatase activity, elevated glucose may possibly induce the inactivation of MAPKs by activating protein tyrosine phosphatases. We also observed that pyruvate stimulates ERK and JNK activities in the presence of RANKL in a synergistic fashion. Despite these findings, the roles of metabolites in RANKL-induced signal activation could not be completely understood until now. In the future, a comprehensive study of metabolite activities in osteoclastogenesis will provide us with more effective solutions for understanding the coordination between metabolic diseases, diabetes and tumors, and bone-related diseases, osteopetrosis and osteoporosis [3-7].

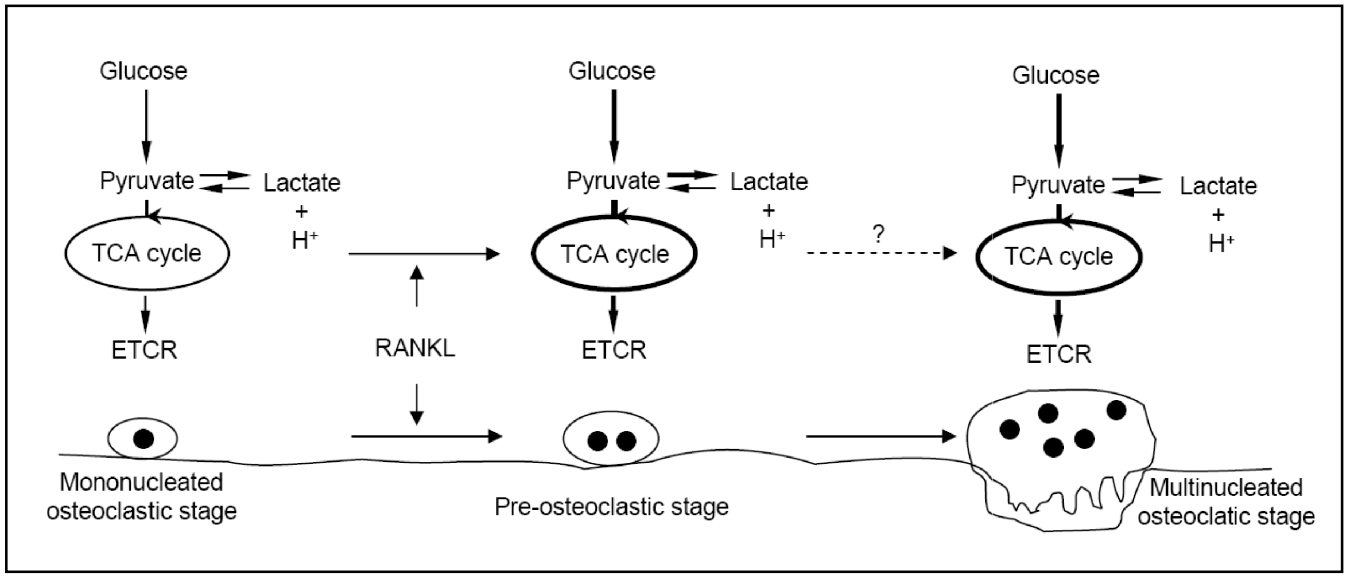


**Fig.10.** Proposed schematic representation for metabolic shifts in the sequential stages of osteoclast differentiation. This schematic illustrates that cells with differential osteoclastic stages have different metabolic flows. TCA cycle, tricarboxylic acid cycle; ETCR, electron transport chain reaction.

Considering that progressively differentiating osteoclasts display metabolic shifts at an early stage after the RANKL stimulation in order to supply efficient production of ATP and biosynthetic intermediates and that this high metabolic ATP production rate promotes osteoclastogenesis, metabolic alterations in these cells must have an important role in the early stages of RANKL-stimulated osteoclast differentiation. A previous study demonstrated that mature osteoclasts at the bone-resorbing stage display enhanced mitochondrial respiration through citric acid cycle and electron transport chain reactions to fulfill high ATP demands, but glycolysis was not effected [32]. Based on our findings and the results from other studies, we propose that metabolic alterations occur in the sequential stages of RANKL-stimulated osteoclast differentiation and bone resorption (Fig. 10). Metabolic shifts provide efficient osteoclastogenesis through a synergistic stimulation of RANKL-induced MAPK activation (ERK and JNK). Further insights into the role of metabolic shifts in the regulation of osteoclastogenesis will continue to help our understanding of bone-related diseases.

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## References

1 Finkel T, Holbrook NJ: Oxidants, oxidative stress and the biology of ageing. Nature 2000;408:239-247.

2 Riggs BL, Melton Iii LJ, 3rd, Robb RA, Camp JJ, Atkinson EJ, Peterson JM, Rouleau PA, McCollough CH, Bouxsein ML, Khosla S: Population-based study of age and sex differences in bone volumetric density, size, geometry, and structure at different skeletal sites. J Bone Miner Res 2004;19:1945-1954.

3 Roodman GD: Mechanisms of bone lesions in multiple myeloma and lymphoma. Cancer 1997;80:1557-1563.

4 Dang CV, Semenza GL: Oncogenic alterations of metabolism. Trends Biochem Sci 1999;24:68-72.

5 Gatenby RA, Gillies RJ: Why do cancers have high aerobic glycolysis? Nat Rev Cancer 2004;4:891-899.
6 Van Poznak C, Sauter NP: Clinical management of osteoporosis in women with a history of breast carcinoma. Cancer 2005;104:443-456.
7 Leidig-Bruckner G, Ziegler R: Diabetes mellitus a risk for osteoporosis? Exp Clin Endocrinol Diabetes 2001;109 Suppl 2:S493-514.
8 Komarova SV, Ataullakhanov FI, Globus RK: Bioenergetics and mitochondrial transmembrane potential during differentiation of cultured osteoblasts. Am J Physiol Cell Physiol 2000;279:C1220-1229.
9 Morrison MS, Turin L, King BF, Burnstock G, Arnett TR: ATP is a potent stimulator of the activation and formation of rodent osteoclasts. J Physiol 1998;511 ( Pt 2):495-500.
10 Francis MJ, Lees RL, Trujillo E, Martin-Vasallo P, Heersche JN, Mobasheri A: ATPase pumps in osteoclasts and osteoblasts. Int J Biochem Cell Biol 2002;34:459-476.
11 Czupalla C, Mansukoski H, Pursche T, Krause E, Hoflack B: Comparative study of protein and mRNA expression during osteoclastogenesis. Proteomics 2005;5:3868-3875.
12 Teitelbaum SL, Ross FP: Genetic regulation of osteoclast development and function. Nat Rev Genet 2003;4:638-649.
13 Hirotani H, Tuohy NA, Woo JT, Stern PH, Clipstone NA: The calcineurin/nuclear factor of activated T cells signaling pathway regulates osteoclastogenesis in RAW264.7 cells. J Biol Chem 2004;279:13984-13992.
14 Kim JM, Kim H, Kwon SB, Lee SY, Chung SC, Jeong DW, Min BM: Intracellular glutathione status regulates mouse bone marrow monocyte-derived macrophage differentiation and phagocytic activity. Biochem Biophys Res Commun 2004;325:101-108.
15 Sauer H, Wartenberg M, Hescheler J: Reactive oxygen species as intracellular messengers during cell growth and differentiation. Cell Physiol Biochem 2001;11:173-186.
16 Jacobson MD: Reactive oxygen species and programmed cell death. Trends Biochem Sci 1996;21:83-86.
17 Yamagata M, Hasuda K, Stamato T, Tannock IF: The contribution of lactic acid to acidification of tumours: studies of variant cells lacking lactate dehydrogenase. Br J Cancer 1998;77:1726-1731.
18 Guidarelli A, Brambilla L, Clementi E, Sciorati C, Cantoni O: Stimulation of oxygen consumption promotes mitochondrial calcium accumulation, a process associated with, and causally linked to, enhanced formation of tert-butylhydroperoxide-induced DNA single-strand breaks. Exp Cell Res 1997;237:176-185.
19 Duzgun SA, Rasque H, Kito H, Azuma N, Li W, Basson MD, Gahtan V, Dudrick SJ, Sumpio BE: Mitogen-activated protein phosphorylation in endothelial cells exposed to hyperosmolar conditions. J Cell Biochem 2000;76:567-571.
20 Zayzafoon M, Botolin S, McCabe LR: P38 and activating transcription factor-2 involvement in osteoblast osmotic response to elevated extracellular glucose. J Biol Chem 2002;277:37212-37218.
21 Nemoto S, Takeda K, Yu ZX, Ferrans VJ, Finkel T: Role for mitochondrial oxidants as regulators of cellular metabolism. Mol Cell Biol 2000;20:7311-7318.
22 Baron R, Neff L, Tran Van P, Nefussi JR, Vignery A: Kinetic and cytochemical identification of osteoclast precursors and their differentiation into multinucleated osteoclasts. Am J Pathol 1986;122:363-378.
23 Miyazaki T, Neff L, Tanaka S, Horne WC, Baron R: Regulation of cytochrome c oxidase activity by c-Src in osteoclasts. J Cell Biol 2003;160:709-718.
24 Dittmann L, Hertz L, Schousboe A, Fosmark H, Sensenbrenner M, Mandel P: Energy metabolism of nerve cells during differentiation. O2 uptake, lactate production and ATP content of chick embryo brain cells before and after cultivation in the Rose chamber. Exp Cell Res 1973;80:425-431.
25 Galons JP, Fantini J, Vion-Dury J, Cozzone PJ, Canioni P: Metabolic changes in undifferentiated and differentiated human colon adenocarcinoma cells studied by multinuclear magnetic resonance spectroscopy. Biochimie 1989;71:949-961.
26 Bax BE, Bloxam DL: Energy metabolism and glycolysis in human placental trophoblast cells during differentiation. Biochim Biophys Acta 1997;1319:283-292.
27 Macheda ML, Rogers S, Best JD: Molecular and cellular regulation of glucose transporter (GLUT) proteins in cancer. J Cell Physiol 2005;202:654-662.
28 Tonack S, Rolletschek A, Wobus AM, Fischer B, Santos AN: Differential expression of glucose transporter isoforms during embryonic stem cell differentiation. Differentiation 2006;74:499-509.
29 Thomas DM, Maher F, Rogers SD, Best JD: Expression and regulation by insulin of GLUT 3 in UMR 106-01, a clonal rat osteosarcoma cell line. Biochem Biophys Res Commun 1996;218:789-793.
30 Ahmed N, Kansara M, Berridge MV: Acute regulation of glucose transport in a monocyte-macrophage cell line: Glut-3 affinity for glucose is enhanced during the respiratory burst. Biochem J 1997;327 ( Pt 2):369-375.
31 Ide R, Maegawa H, Kikkawa R, Shigeta Y, Kashiwagi A: High glucose condition activates protein tyrosine phosphatases and deactivates insulin receptor function in insulin-sensitive rat 1 fibroblasts. Biochem Biophys Res Commun 1994;201:71-77.
32 Dodds RA, Gowen M, Bradbeer JN: Microcytophotometric analysis of human osteoclast metabolism: lack of activity in certain oxidative pathways indicates inability to sustain biosynthesis during resorption. J Histochem Cytochem 1994;42:599-606.