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Bimodal actions of reactive oxygen species in the differentiation and bone-resorbing functions of osteoclasts

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Abstract In order to demonstrate that cellular redox status undergoes decreased reduction during osteoclast differentiation and further decreased reduction during osteoclastic bone resorption, we analyzed γ-glutamylcysteinyl synthetase activity, a glutathione synthesis rate-limiting enzyme, and total glutathione and thiol groups. Moderate and severe redox shifts towards a more oxidizing environment induced gradual increases and decreases in osteoclastogenesis. Moreover, while severe glutathione depletion inhibited bone resorption, moderate glutathione repletion enhanced bone resorption. In summary, our observations suggest that there is a threshold for redox status, representing biphasic patterns in osteoclast differentiation and function.

Keywords: Osteoclast differentiation; Bone resorption; Redox status; Reactive oxygen species; Glutathione

1. Introduction

In aerobic organisms, reactive oxygen species (ROS), such as the superoxide anion and hydrogen peroxide (H_2O_2), are produced intracellularly either as byproducts during mitochondrial electron transport [1] or by the action of several enzymes, including NADPH oxidase [2] and cyclooxygenase [3]. Low levels and transient elevation of endogenously generated ROS are implicated in the regulation of cellular signaling [4]. ROS accumulate in cells as a result of endogenous and exogenous processes and/or due to decreased availability of oxidant defenses. A redox shift toward a more oxidizing environment within cells can damage many cellular constituents [5]. Therefore, ROS play an important role in diverse cellular events, such as proliferation, differentiation, and apoptosis [6–9].

Several studies have recently reported that ROS are associated with bone metabolism and facilitate osteoclast formation and bone resorption [10–13]. We and others have reported that estrogen deficiency in menopause causes a decrease in oxidant defenses [10]. The resulting increase of ROS in the cells could regulate the receptor activator of NF-κB ligand (RANKL)-induced signals that are essential for osteoclast differentiation [14], thus leading to bone loss. NADPH oxidase transiently generates superoxides in RANKL-stimulated osteoclast precursor cells, and this can be inhibited by the addition of exogenous diphenylene iodonium, which, in turn, inhibits osteoclast differentiation [15]. Overexpression of catalase [12,14,16] and glutathione peroxidase [16,17], direct scavengers of H_2O_2, is also known to inhibit osteoclast differentiation. This inhibition indicates that ROS may act as a signal mediator in osteoclast differentiation. We have also reported that cellular reduction status is decreased during osteoclast differentiation [18]. However, the role of altered cellular redox status in osteoclast differentiation is currently unknown.

Our results suggest the possibility of a threshold for a biphasic action of ROS that acts as a signal mediator and oxidative stress response trigger in osteoclast differentiation and bone resorption.

2. Materials and methods

2.1. Macrophage and osteoclast differentiation

Differentiation of bone marrow-derived monocytes (BMMs) into macrophages and osteoclasts was induced as previously described [18]. Briefly, the BMMs prepared from the tibia and femur of 4–6-week-old C57BL6 mice were centrifuged and exposed to hypotonic ACK buffer (0.15 mM NH_4Cl, 1 mM KCO_3, 0.1 mM EDTA, pH 7.4) for 30 s to remove red blood cells. The cells were incubated in a humidified atmosphere of 5% CO_2 at 37 °C in α-MEM (Invitrogen, Carlsbad, CA, USA) containing 10% heat inactivated fetal bovine serum and 5 ng/ml macrophage colony-stimulating factor (M-CSF; Genetics Institute, Cambridge, MA, USA) for 12 h to separate the floating and adherent cells. Floating cells (1×10^5 cells/well in a 24-well culture plate) that were used as BMMs were cultured in the presence of 30 ng/ml M-CSF for 3 days to form macrophage-like osteoclast precursor cells. After floating cells including lymphocytes were removed by aspiration, adherent osteoclast precursors were further incubated in the presence of 30 ng/ml M-CSF and 100 ng/ml RANKL for 4 days to generate osteoclasts. On day 2, the media were replaced with fresh media containing M-CSF and RANKL.

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Abbreviations: ROS, reactive oxygen species; H_2O_2, hydrogen peroxide; RANKL, receptor activator of NF-κB ligand; BMMs, bone marrow-derived monocytes; M-CSF, macrophage colony-stimulating factor; TRAP(+) MNCs, tartrate-resistant acid phosphatase(+) multinucleated cells; γ-GCS, γ-glutamylcysteinyl synthetase; GSH, glutathione; BSO, L-buthionine-(*S*,*R*)-sulfoximine; NAC, *N*-acetylcysteine

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2.2. Tartrate-resistant acid phosphatase (TRAP) staining

After osteoclast differentiation, cells were fixed with 10% formalin and washed with a 1:1 (v/v) mixture of ethanol and acetone. Cells were then stained with Fast Red Violet LB salt (500 μg/ml, Sigma–Aldrich, St. Louis, MO, USA) dissolved in TRAP buffer (120 mM sodium acetate, 66 mM sodium tartrate, pH 5.2, naphthol AS phosphate (100 μg/ml) (all from Sigma–Aldrich)), washed with tap water, air-dried, and photographed under a light microscope. TRAP(+) multi-nucleated cells (TRAP(+) MNCs), containing three or more nuclei, were counted as osteoclasts.

2.3. γ-glutamylcysteinyl synthetase (γ-GCS) activity

Cells were washed with ice-cold PBS, suspended in a buffer (50 mM Tris–HCl, pH 7.4, and protease inhibitor cocktail tablet (Roche, Indianapolis, IN)) and lysed by passing cells through a 25-gauge needle syringe, followed by centrifugation at $20000 \times g$ for 30 min. The cytosolic fractions were used to assess γ-GCS activity, as described [19].

2.4. Total thiol and glutathione (GSH) quantification

The intracellular thiol contents of the cytosolic extracts were determined by DTNB (Sigma–Aldrich) exposure and optical density measurement at 412 nm, as described previously [20]. The total GSH (reduced GSH + oxidized GSSG) was determined, using a colorimetric assay kit for GSH, according to the manufacturer's protocols (BIOXYTECH GSH-412, OXIS International, Portland, OR), with GSH as the standard for calibration.

2.5. Bone resorption assay

BMMs (3×10^4 cells/1 ml/well) were cultured in 24-well OAAS plates (Osteogenic Core Technologies, Korea). After formation of mature osteoclasts, cells were further incubated with or without a redox modifier for two days. Resorption area was measured, according to the manufacture's protocols, using an Image-Pro Plus version 4.5 (Media-Cybernetics®).

3. Results and discussion

3.1. Serial differentiation of BMMs into macrophages and osteoclasts

BMMs were differentiated into macrophage-like osteoclast precursor cells in the presence of M-CSF and then into osteoclasts by culturing in the presence of M-CSF and RANKL. Macrophages showed macrophage characteristics, including a time-dependent increase in cell adhesion (Fig. 1A, upper panel, and Fig. 1C, left panel) and phagocytic capacity (Fig. 1A, middle panel, and Fig. 1B). It was also observed that TRAP(+) MNCs, characteristic of osteoclasts, increased gradually during differentiation (Fig. 1A, lower panel, and Fig. 1C, right panel). These results indicated that the serial differentiation of BMMs into macrophages and then into osteoclasts was successfully achieved in our model system.

3.2. Changes of cellular redox status in macrophage and osteoclast differentiation

Glutathione (GSH) plays a pivotal role in oxidant defense by catabolizing H_2O_2 and other peroxides through an enzymatic

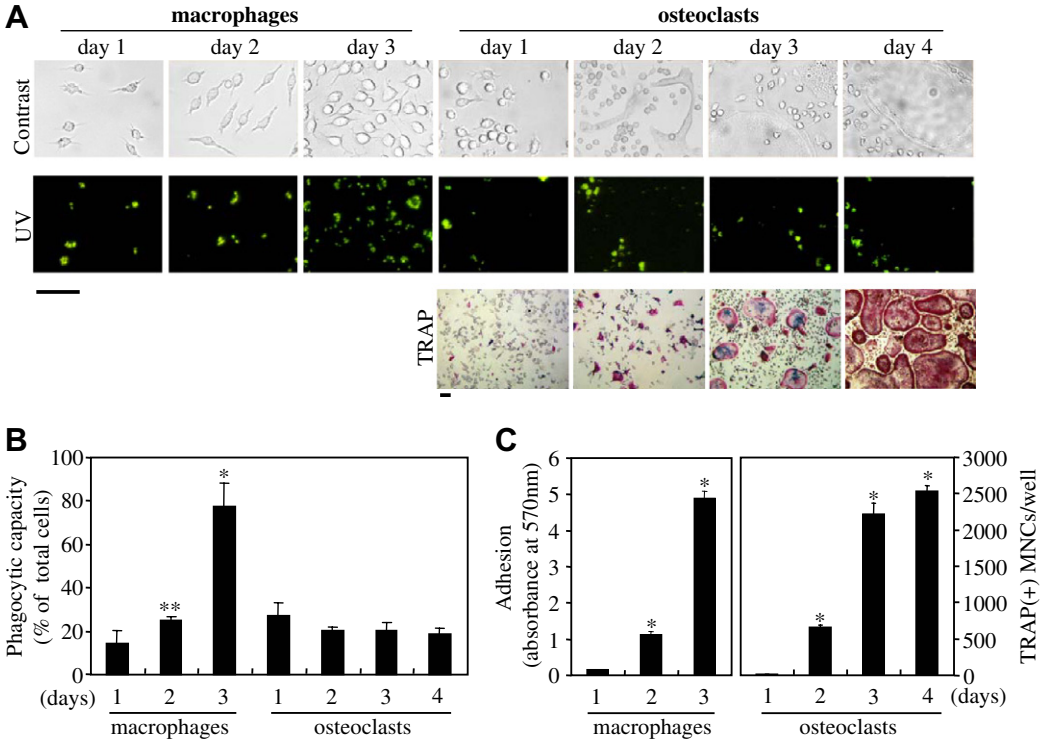

Fig. 1. Characteristics of macrophages and osteoclasts during serial differentiation from BMMs. (A) Phagocytic activity and TRAP staining. After macrophage and osteoclast differentiation, cells were incubated with 10 μg/ml fluorescein-conjugated zymosan A Bio Particle (Molecular Probes, Eugene, OR) for 1 h, washed with PBS three times, added to PBS, and photographed using a fluorescence microscope (Olympus B×51 microscope) under visible (upper panel) and UV illumination (middle panel). Cells were also stained for TRAP to detect osteoclasts (lower panel). Bar, 50 μm. (B) Changes of the phagocytic activity in macrophage and osteoclast differentiation. Phagocytic activity was calculated as a percentage of macrophages and osteoclasts that incorporated the zymosan particles per total cells. Data are expressed as means ± S.D. of triplicates. $^{*}P < 0.01$; $^{**}P < 0.05$ vs. day 1 macrophages. (C) Adhesion assay and the number of TRAP-positive cells. The adherent cells were washed with PBS, fixed with 10% formalin, and stained with 0.5% crystal violet. After washing with tap water, the stained cells were dissolved with a 2% SDS solution and the absorbance was measured spectrophotometrically at 570 nm (left panel). After TRAP staining, TRAP(+) MNCs (>3 nuclei) were counted as osteoclasts (right panel).

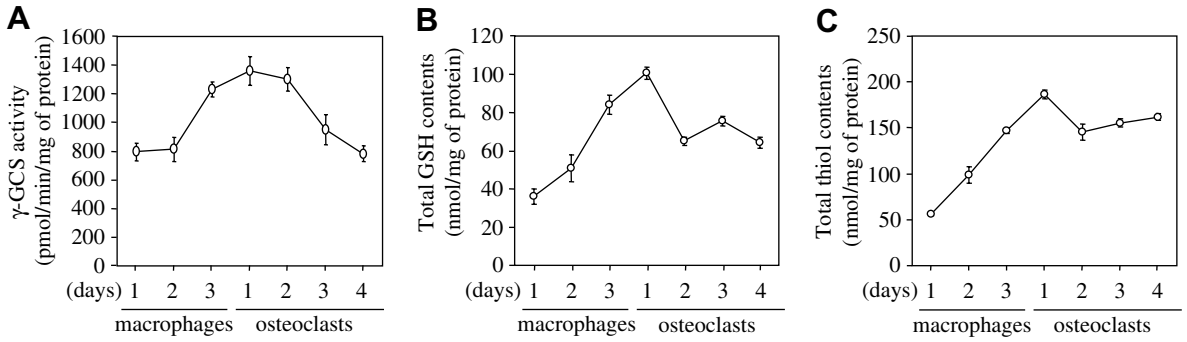

Fig. 2. Redox status fluctuation and γ-GCS activity during differentiation of bone marrow-derived macrophages and osteoclasts. BMMs (1×10^6 cells) were seeded on a 60-mm culture plate and harvested at 1-day intervals after M-CSF-induced macrophage differentiation and M-CSF plus RANKL-induced osteoclast differentiation. (A) γ-GCS activity in differentiation of macrophages and osteoclasts. (B and C) Changes of total GSH and thiol groups. Data are expressed as means ± S.D. of triplicates.

coupling reaction, thus protecting protein thiol groups from oxidation. GSH contributes to the maintenance of almost 90% of total cellular redox status [21,22]. Therefore, we first measured the activities of γ-GCS, which is a GSH synthesis rate-limiting, heterodimeric enzyme, composed of catalytic and modifier subunits [23]. γ-GCS activity gradually increased during macrophage differentiation and sequentially decreased during osteoclast differentiation (Fig. 2A). This finding is consistent with previous data, showing that the reduced GSH/oxidized GSSG ratio declines and the total GSH content decreases during differentiation and that these events are dependent on down-regulation of the γ-GCS modifier subunit gene [18]. To confirm this, it was also observed that total GSH content and cellular thiol groups present were similar to the pattern of γ-GCS activity observed during differentiation (Fig. 2B and C). Thus, our findings show that redox status fluctuates during the serial differentiation of BMMs → macrophages → osteoclasts, and is tightly dependent on GSH synthesis.

3.3. Effects of cellular redox status on osteoclast differentiation

There exists evidence that ROS, generated by RANKL-stimulated NADPH oxidase, are associated with osteoclast differentiation [14,15]. We also observed that H_2O_2 alone and the combination of H_2O_2 and RANKL directly and synergistically stimulate RANKL-induced signals (Fig. 3A), including ERK, JNK, p38, and transcription factor NF-κB, which are essential for osteoclastogenesis. Since extreme oxidative stress could cause cell death, we reconsidered the possibility that ROS may modify biological functions of cells when redox status changes are still within the limits of cell tolerance. Although treatment with H_2O_2 and L-buthionine-(*S*,*R*)-sulfoximine (BSO) at the indicated concentrations in the present study induced a more oxidizing status, the redox modifiers themselves did not have any effect on cell viability (data not shown). Change in redox status, induced by varying the concentration of H_2O_2 (0–40 μM) and BSO (0–300 μM), a specific inhibitor of GSH synthesis, showed a biphasic pattern in osteoclastogenesis (Fig. 3B and C). While moderate concentrations (<1 μM in H_2O_2 and <10 μM in BSO) enhanced formation of TRAP(+) MNCs, excessive concentrations (>1 μM in H_2O_2 and >10 μM in BSO) decreased the number of TRAP(+) MNCs. One possible interpretation is that a redox shift occurs during differentiation events through a combination of exogenous H_2O_2- and BSO-mediated thiol depletion in conjunction with decreased reduction status in RANKL-induced differentiation.

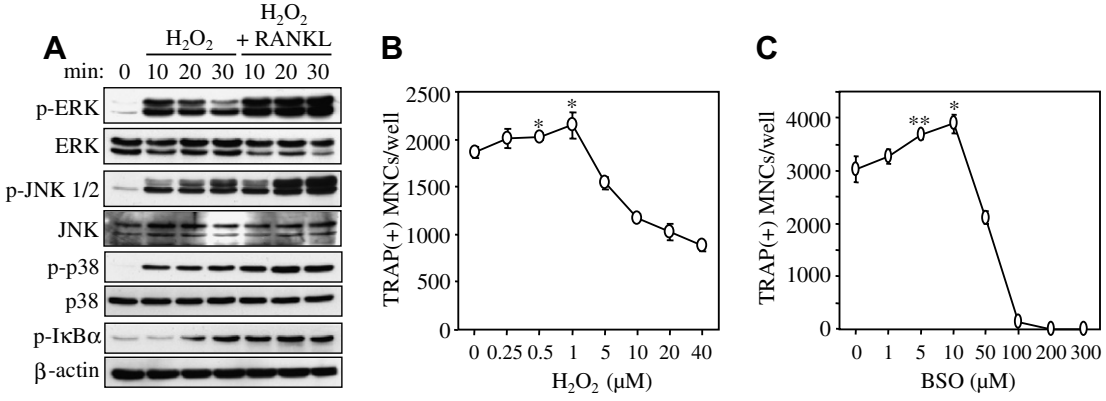

Fig. 3. Effects of redox modifiers on osteoclast differentiation. (A) Synergistic effects of exogenous H_2O_2 on RANKL-induced signals. Macrophages (osteoclast precursors) were stimulated with H_2O_2 (1 mM) or H_2O_2 plus RANKL (100 ng/ml) for 10 min in the presence of M-CSF. Whole extracts were subjected to immunoblot analysis, using specific antibodies to p-ERK, ERK, p-JNK, JNK, p-p38, p38, p-IκBα, and β-actin. (B and C) Effects of H_2O_2 and BSO on osteoclast differentiation. Macrophages were differentiated into osteoclasts by incubation with M-CSF and RANKL in the presence of H_2O_2 (0–40 μM) or BSO (0–300 μM). After incubating for 4 days, cells were stained with TRAP and the TRAP(+) MNCs were counted. Data are expressed as means ± S.D. ($n = 3$). $^{*}P < 0.01$; $^{**}P < 0.05$ vs. untreated control.

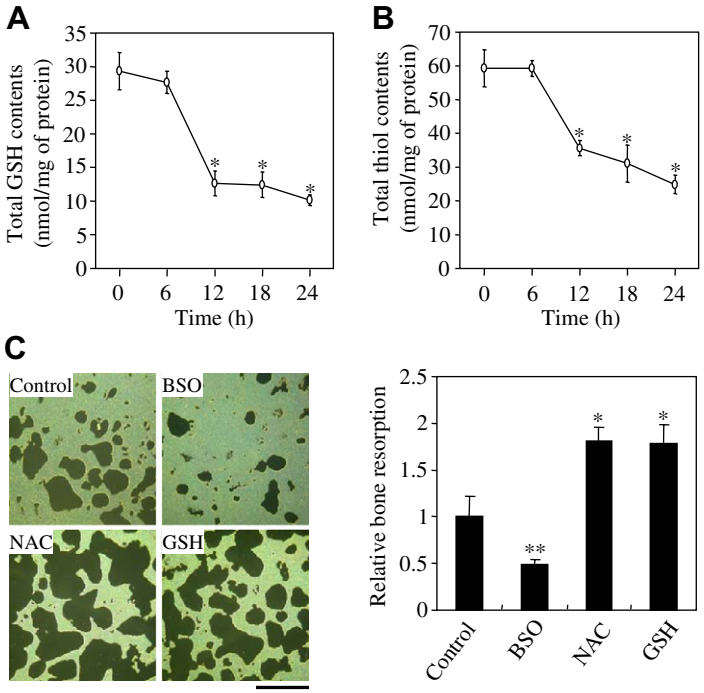

Fig. 4. Effects of redox modifiers on bone resorption. (A and B) Change of redox status in mature osteoclasts. After the formation of mature osteoclasts, total GSH and thiol groups were measured at the indicated time intervals during bone resorption. Data are expressed as means ± S.D. of triplicates. $^{*}P < 0.01$ vs. 0 h. (C) Bone resorption by redox modifiers. Mature osteoclasts, cultured in 24-well OAAS plates, were incubated with BSO (300 μM), NAC (5 mM) or GSH (5 mM) for 2 days in the presence of M-CSF and RANKL prior to assessment of the area of bone resorption. Data are expressed as means ± S.D. of triplicates of one of three independent experiments. Bar, 100 μm. $^{*}P < 0.01$; $^{**}P < 0.05$ vs. control.

3.4. Effects of an oxidizing or reducing cellular redox status on bone resorption

The cellular reduction status at the bone-resorbing stage is still less than that at day 4 after osteoclast differentiation (Fig. 4A and B vs. Fig. 2B and C). As seen previously, ROS, such as the superoxide anion and hydrogen peroxide, are associated with enhanced bone resorption [12,13]. Conversely, in the present study, when mature osteoclasts, that are continually undergoing bone resorption, were treated with BSO (300 μM) for 2 days, the total GSH was decreased more than 10-fold, resulting in a lower bone resorption than the untreated control (Fig. 4C). This is likely to result from an oxidative stress environment, caused by the deleterious combination of an oxidizing environment created by BSO and a low reduction status during the period of bone resorption. Interestingly, bone resorption was facilitated by the addition of *N*-acetylcysteine (NAC), a GSH precursor, and reduced GSH. This stimulation may be due to enhancement of redox capacity with GSH repletion to a level sufficient to overcome a low reduction status at the bone-resorbing stage. Treatment of macrophages or mature osteoclasts with the indicated redox compounds and concentrations affected neither cell viability nor proliferation (data not shown). This result suggests that changes in osteoclast differentiation and function, seen after treatment with redox modifiers, are not likely due to the effects of cell damage.

4. Conclusions

Based on findings from our experiments and others, we propose the following possible actions for ROS in osteoclastogenesis and bone resorption: (i) the decline in oxidant defense during osteoclast differentiation and bone resorption results in a decrease in cellular reduction status; (ii) during high reduction status in osteoclast precursor macrophages (prior to differentiation), the cellular environment maintains enhanced sensitivity to small-scale ROS oscillations; (iii) ROS, transiently generated by RANKL-induced NADPH oxidase activation at early stages of osteoclast differentiation, stimulates differentiation-related signals, suggesting that ROS acts as a signal mediator; (iv) lowered reduction status, which occurs during the late stages of differentiation and bone-resorbing stages of mature osteoclasts, may function as an oxidative stressor to delay osteoclast differentiation and bone resorption.

Collectively, we show here that osteoclast differentiation and function may be positively or negatively regulated by cellular redox status. Further, we propose that ROS function bimodally as cell signal mediators and oxidative stressors in osteoclast differentiation and bone resorption.

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