ORIGINAL

NADPH oxidase-derived reactive oxygen species are essential for differentiation of a mouse macrophage cell line (RAW264.7) into osteoclasts

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Abstract : Reactive oxygen species (ROS) derived from NADPH oxidase (Nox) homologues have been suggested to regulate osteoclast differentiation. However, no bone abnormalities have been documented in Nox1 deficient, Nox2 deficient, or Nox3 mutant mice. During receptor activator of nuclear factor-κB ligand (RANKL)-stimulated differentiation of a mouse macrophage cell line (RAW264.7) into osteoclasts, mRNA levels of Nox enzymes (Nox1-4) and their adaptor proteins were monitored by real-time reverse transcriptase PCR. RAW264.7 cells constitutively expressed abundant Nox2 mRNA and small amounts of Nox1 and Nox3 transcripts. RANKL markedly attenuated Nox2 mRNA expression in association with reciprocal up-regulation of Nox1 and Nox3 transcripts. Introduction of small interference RNA targeting $p67^{phox}$ or $p22^{phox}$ into RAW264.7 cells effectively down-regulated ROS generation and significantly suppressed the RANKL-stimulated differentiation, which was assessed by appearance of tartrate resistant acid phosphatase (TRAP)-positive, multinucleated cells having an ability to form resorption pits on calcium phosphate thin film-coated disks, and by expression of osteoclast marker genes (TRAP, cathepsin K, Atp6i, ClC-7, and NFATc1). Our results suggest that RANKL may stimulate switching between Nox homologues during osteoclast differentiation, and Nox-derived ROS may be crucial for RANKL-induced osteoclast differentiation. J. Med. Invest. 56 : 33-41, February, 2009

Keywords **:** *osteoclasts, differentiation, bone resorption, reactive oxygen species, NADPH oxidase*

INTRODUCTION

Osteoclasts are a member of the monocyte/macrophage lineage and are formed by multiple cellular fusions from their mononuclear precursors. They play an important role in bone metabolism by regulating bone resorption. Receptor activator of nuclear factor-κB ligand (RANKL) induces differentiation into osteoclasts from myeloid precursors at various intermediate stages as well as from well differentiated tissue macrophages such as alveolar macrophages (1). One of the characteristic features of this lineage is the presence of the phagocyte NADPH oxidase (2-5). Superoxide anion ($O_2^{\cdot-}$) and related reactive oxygen species (ROS) produced by the phagocyte NADPH oxidase play a crucial role in the process of killing ingested microorganisms (6). The phagocyte NADPH oxidase consists of a

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membrane-integrated flavocytochrome b_{558}, composed of gp91^{phox} and p22^{phox}, and four cytosolic components (p47^{phox}, p67^{phox}, p40^{phox}, and Rac) that associate with the flavocytochrome to form an active enzyme (6).

Recently, two families of gp91^{phox} homologues have been identified as potential sources of ROS: NADPH oxidase (Nox) and dual oxidase (Duox) families (7, 8). The Nox family comprises Nox1, Nox2 (gp91^{phox}), Nox3, Nox4, and Nox5. Nox1 is also associated with the membrane-integrated protein p22^{phox} and requires at least three additional cofactors, Nox organizer 1 (NOXO1), Nox activator 1 (NOXA1), and Rac1 for the activation of the Nox1-based oxidase system (7, 8). Nox1 is also able to use the p47^{phox} and p67^{phox} subunits (9). Nox3 is a p22^{phox}-dependent enzyme and is constitutively inactive, weakly active, or substantially active depending on cell type (7, 8). NOXO1 enhances Nox3 activity. However, the requirement for the other components is still contradictory. Nox4 is also a p22^{phox}-dependent enzyme (10). According to our present knowledge, Nox4 does not require cytosolic subunits. These new enzymes show tissue- and cell type-specific distribution and are proposed to have distinct functions (7, 8).

Osteoclasts attach to the surface of bone and secrete protons into an extracellular compartment between osteoclast and bone surface. The osteoclast proton pump is essential for bone mineral solubilization and digestion of organic bone matrix by acid proteases (11). At the same time, ROS produced by activated osteoclast are suggested to participate in the complex process of bone resorption (2, 4). It has also been suggested that ROS derived from osteoclast precursors are essential for osteoclast differentiation (12). Osteoclasts express Nox2 in their ruffled borders (5). Considering the origin of osteoclasts, it is conceivable that Nox2 may be an enzyme responsible for the ROS-dependent differentiation and bone-resorbing activities. However, it is known that Nox2 knockout (Nox2$^{-/-}$) mouse osteoclasts are still able to produce $O_2^{\cdot-}$ (13), and no bone abnormalities have been documented in Nox2$^{-/-}$ mice or in patients with chronic granulomatous disease. These observations could be explained at least in part by the expression of Nox4 mRNA and protein in mature osteoclasts (13, 14) and Nox1 in osteoclast precursors (12). In fact, Nox4 was reported to be responsible for generation of $O_2^{\cdot-}$ and formation of resorption pits by osteoclasts (13, 14). A recent study has suggest that Nox1 rather than Nox2 or Nox4 is important for RANKL-initiated ROS production and differentiation into osteoclasts (12). However, even in the case of Nox1$^{-/-}$ mice, no bone abnormalities have so far been reported (15, 16). Although there may be an active compensatory mechanism for the Nox system in the osteoclast lineage, it is still unclear whether Nox-derived ROS are essential for osteoclast differentiation.

In this study, using a mouse monocyte cell line (RAW264.7), we examined the role of Nox homologues in RANKL-induced differentiation into osteoclasts and bone-resorbing activity.

MATERIALS AND METHODS

1. Reagents

Recombinant mouse RANKL was prepared as described previously (17). Histopaque-1077, naphthol AS-MX phosphate, fast red violet LB salt, phorbol 12-myristate 13-acetate (PMA), and superoxide dismutase (SOD) were obtained from Sigma Chemical Co. (St. Louis, MO). Submicron synthesis calcium phosphate thin film-coated disks were purchased from BD Bioscience (Bedford, MA). The enhancer-containing luminol-based detection system (Diogenes) was obtained from National Diagnostics (Atlanta, GA). A rabbit anti-serum against human Nox2 (residues 536-555) was a gift from Dr. Tsunawaki, National Research Institute for Child Health and Development, Tokyo.

2. Culture of RAW264.7 (RAW) cells

RAW cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal calf serum (FCS; Invitrogen, Carlsbad, CA), 100 U/ml penicillin G, and 100 μg/ml streptomycin at 37°C under 95%-air and 5%-CO_2. To stimulate differentiation of RAW cells into osteoclasts, they were cultured with RANKL (150 ng/ml) in phenol red-free α-minimum essential medium (α-MEM) containing 10% heat-inactivated FCS in 12-well (1×10^4 cells/well), 24-well ($0.5\text{-}1\times10^4$ cells/well), 48-well (3×10^3 cells/well), or 96-well (1×10^3 cells/well) culture plates. A half volume of the RANKL-containing medium was replaced with the fresh medium every 3 days.

3. Real-time reverse transcriptase (RT)-PCR

Total RNA was extracted using an acid guanidium-thiocyanate-phenol chloroform mixture (18). Levels of Nox1, Nox2, Nox3, Nox4, p47^{phox}, p67^{phox}, p22^{phox},

NOXO1, NOXA1, Rac1, Rac2, TRAP, cathepsin K, ATPase (Atp6i), chloride channel 7 (ClC-7), nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs were measured by quantitative real-time reverse transcriptase (RT)-PCR. Specific primer sets for Nox1 (ABI part No.Mm01340621_g1), Nox2 (Mm00432774_m1), Nox3 (Mm01339126_m1), Nox4 (Mm00479246_m1), $p47^{phox}$ (Mm00447921_m1), $p67^{phox}$ (Mm00726636_s1), $p22^{phox}$ (Mm00514478_m1), NOXO1 (Mm00546832_g1), NOXA1 (Mm00549171_m1), Rac1 (Mm01201653_mH), Rac2 (Mm00485472_m1), TRAP (Mm00475698_m1), cathepsin K (Mm00484035_m1), Atp6i (Mm00469395_g1), ClC-7 (Mm00442400_m1), NFATc1 (Mm00479445_m1), and GAPDH (Mm99999915_g1) were designed using the Primer Express program (Applied Biosystems, Foster City, CA). cDNA was generated from 1 μg of total RNA with Multiscribe Reverse Transcriptase (Applied Biosystems) using oligodT/hexamer primers. Real-time RT-PCR was performed using the ABI 7500 (Applied Biosystems). Data were normalized for the amount of GAPDH mRNA.

4. Western blot analysis

Membrane proteins were prepared from RAW cells and peritoneal macrophages from $Nox2^{-/-}$ mice (provided by Mary C. Dinauer, Indiana University School of Medicine, Indianapolis, IN, USA) (19) as previously described (18), and amounts of Nox2 in the membrane fractions were measured by immunoblot analysis using antiserum against the residues 536-555 of human Nox2 (20).

5. Tartrate resistant acid phosphatase (TRAP) stain

Cells were stained for TRAP activity as previously described (17). Cells were fixed with 10% formalin in phosphate-buffered saline at room temperature for 15 min. They were quenched with a 1 : 1 (v/v) mixture of ice cold acetone and ethanol. These cells were incubated for 15-60 min at 37°C with 0.1 M acetate buffer (pH 5), containing 25 mM sodium tartrate, 0.01% naphthol AS-MX phosphate, and 0.06% fast red violet LB salt, and then rinsed with water. TRAP-positive multinucleated cells having≥three nuclei were counted as osteoclasts under microscopic observation.

6. Bone resorption assay

Bone-resorbing activity was assessed using submicron synthesis calcium phosphate thin film-coated disks. RAW cells were cultured with 150 ng/ml RANKL for the indicated days on the calcium phosphate thin film-coated disks in 24-well culture plates. After adherent cells were removed by washing with 5% sodium hypochlorite for 5 min, the disk was washed with water and photographed. The area of resorption pits on the disk was measured in 16 randomly selected areas and analyzed with NIH imageJ software (http : //rsb.info.nih.gov/ij/).

7. Measurement of $O_2^{\cdot-}$

The rate of $O_2^{\cdot-}$ production by RAW cells and RAW cell-derived osteoclasts was determined by SOD-inhibitable chemiluminescence using an enhancer-containing luminol-based detection system (Diogenes ; National Diagnostics). RAW cells were left untreated or treated with 150 ng/ml RANKL for 5 days. These cells were incubated with the enhanced luminol-based substrate (50% of total reaction volume) in Hank's balanced salt solution (HBSS) at 1×10^5 cells/well in 96-well clear-bottom white plates (Nunc Inc., Naperville, IL). Both spontaneous and 50 ng/ml PMA-stimulated changes in chemiluminescence were measured in the presence or absence of 20 μg/ml SOD with a microplate reader (Wallac 1420 ARVO™MX ; PerkinElmer, Waltham, MA) and were expressed in arbitrary units during 1-s readings recorded at 1-min intervals over a time course of 30 min.

8. Small interfering RNA (siRNA)

A duplexed Stealth™ siRNA designed against $p22^{phox}$ (accession no. NM_007806) or $p67^{phox}$ (accession no. NM_010877) was purchased from Invitrogen. The sequences were as follows : $p22^{phox}$ siRNA, 5'-CCAGGUUAACCCAAUGCCAGUGACA-3' (sense) and 5'-UGUCACUGGCAUUGGGUUAACCUGG-3' (antisense) ; $p67^{phox}$ siRNA, 5'-CCAUGGAGAGCAUCUGGAAGCAGAA-3' (sense) and 5'-UUCUGCUUCCAGAUGCUCUCCAUGG-3' (antisense). RAW cells were seeded at a density of 5×10^5 cells/dish in 35-mm-diameter culture dishes or 1×10^5 cells/well in 24-well culture plates and transfected with control siRNA (Stealth RNAi negative control, Invitrogen), $p22^{phox}$ siRNA, or $p67^{phox}$ siRNA using Lipofectamine RNAiMAX (Invitrogen) for 48 h according to the manufacturer's protocol. After transfection, these cells were collected and cultured in 24- or 96-well plates with 150 ng/ml RANKL in α-MEM supplemented with 10% FCS for 5 days.

RESULTS

1. Effects of RANKL treatment on mRNA expression of Nox enzymes and their regulatory proteins in RAW cells

As shown in Fig. 1, RAW cells constitutively expressed Nox2, $p47^{phox}$, NOXO1, $p67^{phox}$, $p22^{phox}$, Rac1, and Rac2 mRNAs. Small amounts of Nox1 (about 0.005% of Nox2 mRNA level) and Nox3 (about 0.001% of Nox2 mRNA level) mRNAs could be detected, while RAW cells did not express Nox4 or NOXA1 transcripts. RANKL treatment dramatically decreased the Nox2 mRNA level to around 2% of the original level within 3 days (Fig. 1A). This decline was associated with the reduction of $p47^{phox}$ (Fig. 1 D) and $p67^{phox}$ (Fig. 1E) mRNA levels. In contrast, RANKL inversely up-regulated expression of Nox1 about 8-fold (Fig. 1B). However, expression of its organizer protein (NOXO1) mRNA was rather down-regulated (Fig. 1F). Nox3 mRNA level gradually increased after treatment with RANKL (Fig. 1C), but its level was still around 10% of that of Nox1 mRNA on day 5. We also confirmed that Nox4 transcript was not detectable even after stimulation by RANKL (data not shown).

2. Reduction of Nox2 protein in RAW cells after treatment with RANKL

A rabbit anti-serum against human Nox2 (residues 536-555) was shown to recognized Nox2 protein, preferentially its unglycosylated form (20). To confirm the specificity of the antiserum, membrane proteins were prepared from peritoneal macrophages of $Nox2^{-/-}$ mice and used as a negative control.

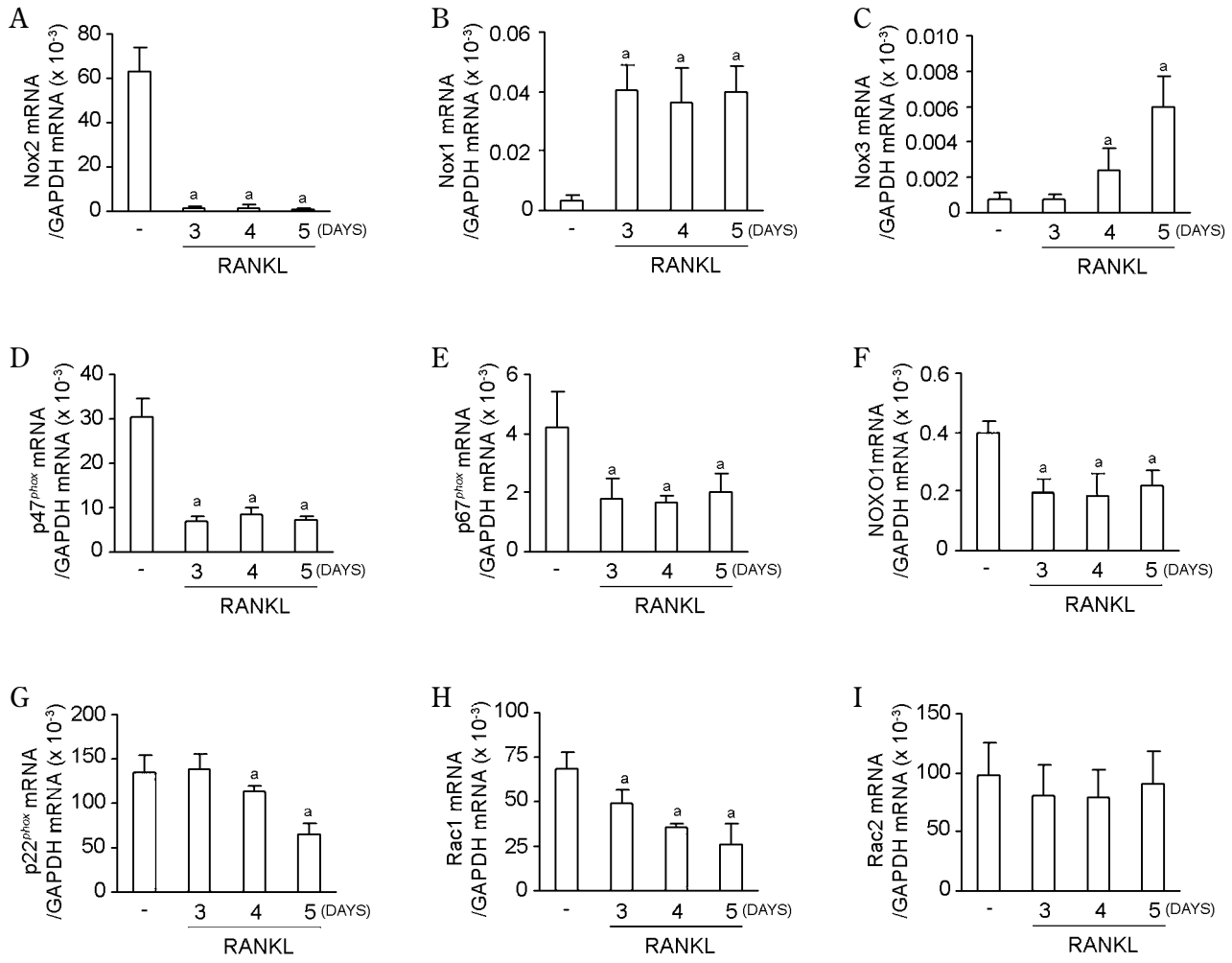

Fig. 1. Expression of mRNAs for Nox enzymes and their adaptor proteins in RANKL-treated RAW cells.
RAW cells were left untreated or treated with 150 ng/ml RANKL for the indicated days. A half volume of the medium was replaced with the fresh medium every 3 days. (A-I) Total RNA was prepared from these cells, and mRNA levels of Nox1, Nox2, Nox3, $p22^{phox}$, $p47^{phox}$, $p67^{phox}$, NOXO1, Rac1, and Rac2 were measured by real-time RT-PCR. GAPDH mRNA level was used as an internal quantity control. Values are means ± SD in three independents experiments. [a]Significantly different, compared with untreated control cells ($P < 0.05$ by ANOVA and Scheffé's test).

Western blotting showed that the anti-Nox2 serum mainly recognized 63-kDa protein and several proteins with higher molecular weights, and these proteins were absent in peritoneal macrophages from Nox2 knockout mice (Fig. 2). Consistent with the marked decline of Nox2 mRNA level, Nox2 protein level was substantially decreased in RAW cell-derived osteoclasts.

3. RANKL-induced differentiation of RAW cells into osteoclasts

After treatment with RANKL, RAW cells gradually changed their shapes, and TRAP-positive, multinucleated cells appeared on day 3 and constituted 70-80% of total cells on day 5 (Fig. 3A). These cells exhibited bone resorbing activity (Fig. 3A). We also measured mRNA expression of genes associated with osteoclastogenesis. Treatment of RAW cells with RANKL up-regulated expression of TRAP (Fig. 3B), Cathepsin K (Fig. 3C), Atp6i (Fig. 3D), and ClC-7 (Fig. 3E) mRNAs during the differentiation into osteoclasts.

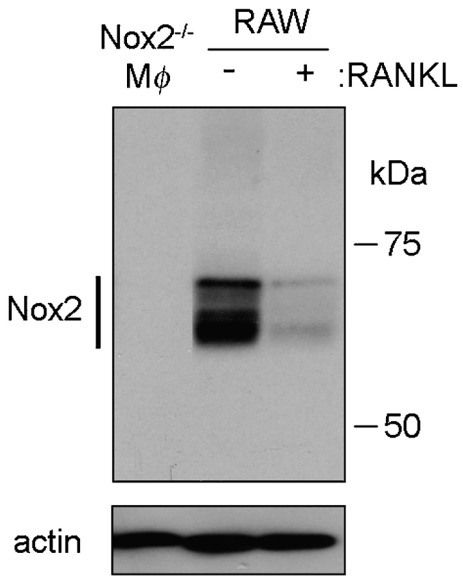

Fig. 2. RANKL-induced decline of Nox2 protein in RAW cells. Membrane proteins were prepared from peritoneal macrophages (Mϕ) from Nox2$^{-/-}$ mice, RAW cells, and RAW cell-derived osteoclasts, as previously described (18), and amounts of Nox2 in the membrane proteins (2 μg protein per lane) were measured by immunoblot analysis using antiserum against the residues 536-555 of human Nox2 (20).

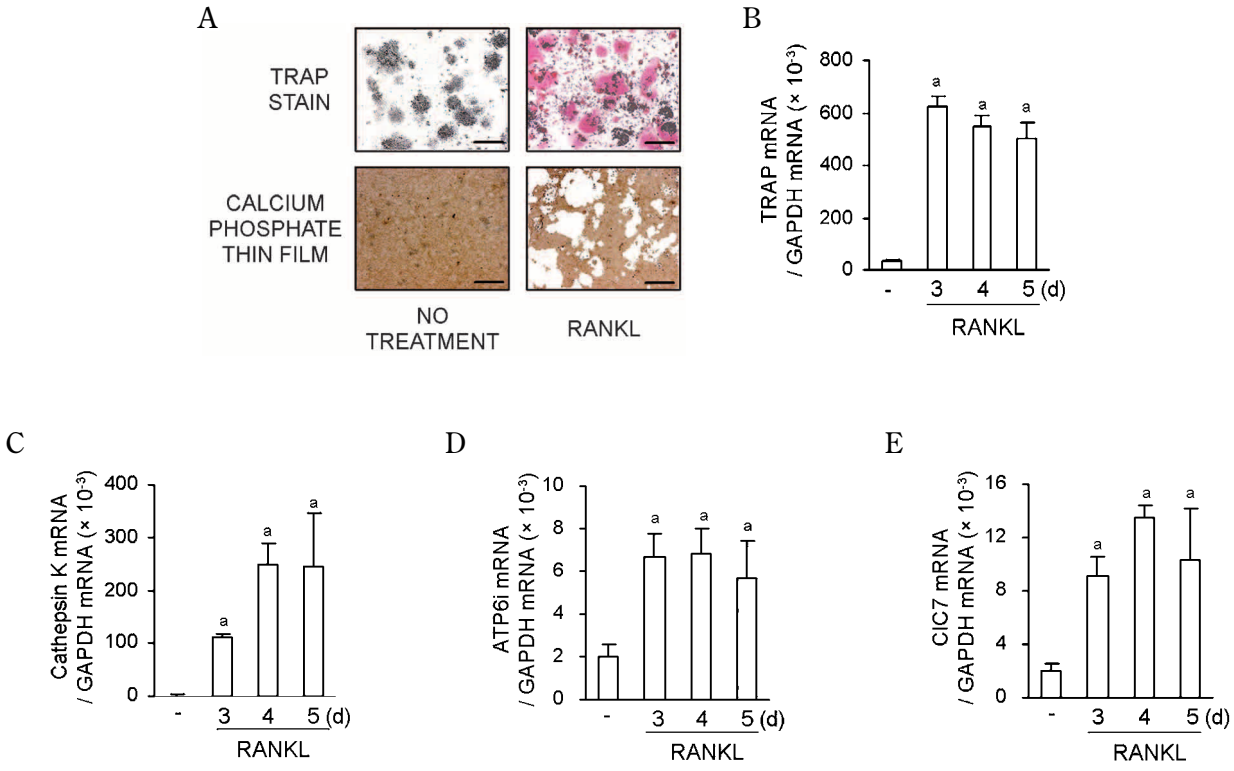

Fig. 3. RANKL-induced differentiation of RAW cells into osteoclasts.
(A) RAW cells left untreated or treated with 150 ng/ml RANKL for 5 days were fixed and stained with TRAP on day 5 (upper panels). RAW cells were cultured for 5 days on submicron synthesis calcium phosphate thin films in the presence or absence of 150 ng/ml RANKL. After removal of the cells, the thin films were photographed using light microscopy (lower panels). Scale bars are 400 μm. Levels of TRAP (B), Cathepsin K (C), Atp6i (D), and ClC-7 (E) mRNAs were measured by real-time RT-PCR using GAPDH mRNA level as an internal quantity control. Values are means ± SD of four independent experiments. [a]Significantly different, compared with untreated control cells ($P < 0.05$ by ANOVA and Scheffé's test).

4. $O_2^{\cdot-}$ production by RAW cells and RAW cell-derived osteoclasts

We also examined how RANKL treatment changed $O_2^{\cdot-}$-producing capability in RAW cells. In association with the striking changes in Nox homologue expression, particularly with the marked decline of Nox2 expression, spontaneous and PMA-stimulated $O_2^{\cdot-}$ production by RAW cell-derived osteoclasts decreased to about 8% and 3% of the respective control values (Figs. 4A and 4B).

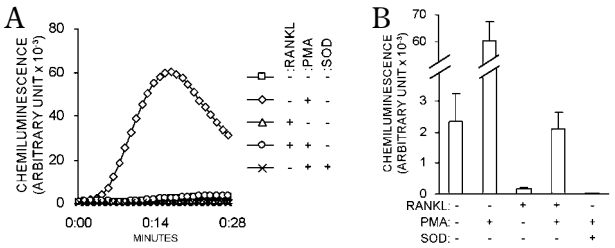

Fig. 4. Changes in $O_2^{\cdot-}$ production by RAW cells after treatment with RANKL.
(A) After RAW cells were cultured with or without 150 ng/ml RANKL for 5 days, chemiluminescence derived from spontaneous or PMA-stimulated $O_2^{\cdot-}$ production by these cells was continuously monitored with an enhanced luminol-based substrate, Diogenes, in the presence or absence of 20 μg/ml SOD. (B) Each value is mean ± SD (n=4) of the peak chemiluminescence values.

5. Effects of knockdown of $p22^{phox}$ or $p67^{phox}$ in RAW cells on osteoclast differentiation

No bone abnormalities have been documented in $Nox2^{-/-}$ (13) and $Nox1^{-/-}$ mice (15, 16). Mutation in the Nox3 gene has defect in otoconia morphogenesis, while no bone abnormalities have been documented in the mutant mice (21). RAW cells did not express Nox4, but could differentiate into osteoclasts in response to RANKL. These observations suggest that there may be a flexible compensatory mechanism among the Nox family to maintain ROS generation during osteoclast differentiation.

Using RAW cells, we examined whether Nox-derived ROS were essential for osteoclast differentiation. RAW cells expressed only $p67^{phox}$ as a Nox activator and did not express Nox4. Therefore, it was considered that knockdown of $p67^{phox}$ or $p22^{phox}$ effectively suppressed the $O_2^{\cdot-}$-producing capability of Nox enzymes expressed in RAW cells. RAW cells were treated with different concentrations (10-100 nM) of siRNA against $p67^{phox}$ or $p22^{phox}$ for 48 h. As shown in Fig. 5, 50 nM $p22^{phox}$ siRNA (Fig. 5A) and 50 nM $p67^{phox}$ siRNA (Fig. 5B) knocked down respective mRNA levels to < 30%, and consequently PMA-stimulated $O_2^{\cdot-}$ generation was decreased to

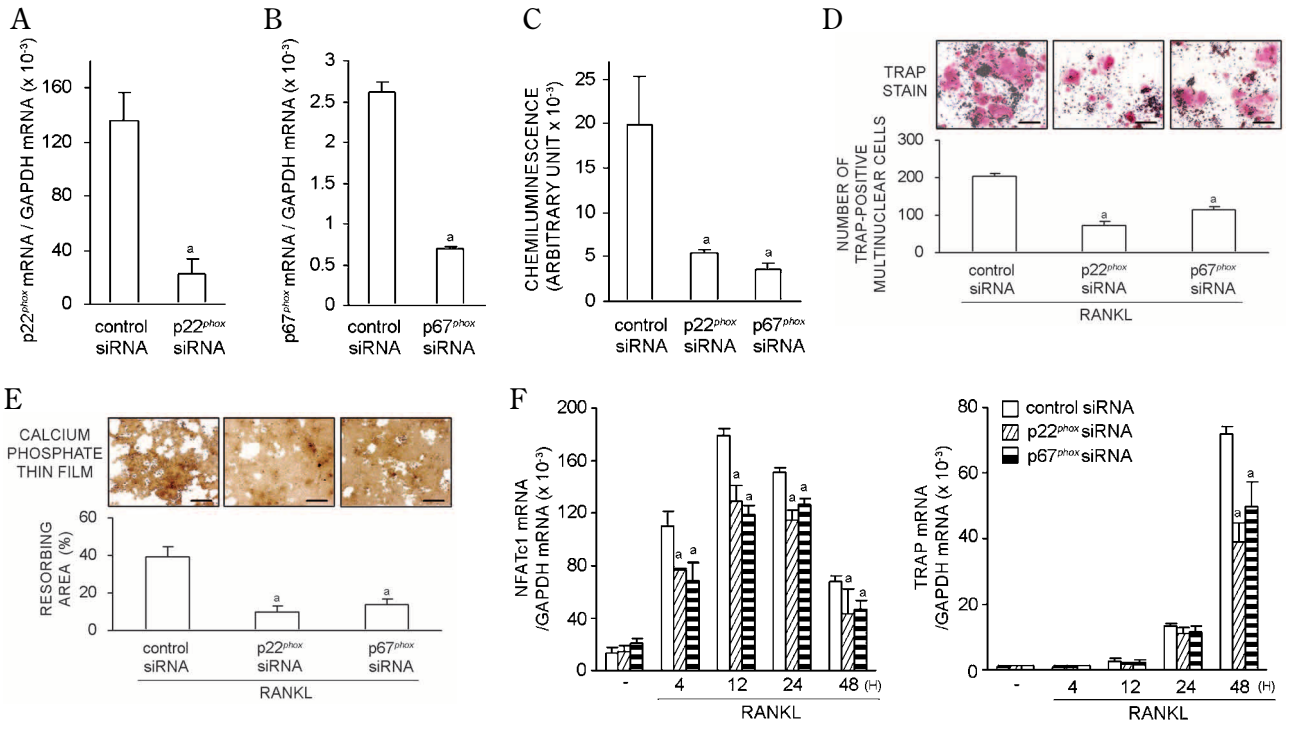

Fig. 5. Effects of $p22^{phox}$ or $p67^{phox}$ siRNA on differentiation of RAW cells into osteoclasts.
After RAW cells were transfected with 50 nM $p22^{phox}$ siRNA, 50 nM $p67^{phox}$, or 50 nM control siRNA (Stealth RNAi negative control ; Invitrogen), they were cultured with 150 ng/ml RANKL for 5 days. $p22^{phox}$ mRNA levels (A), $p67^{phox}$ mRNA levels (B), PMA-stimulated $O_2^{\cdot-}$ generation (C), TRAP-positive cells (D), bone resorption (E), and NFATc1 and TRAP mRNA levels (F) were measured as described in the legends to the above Figs. Scale bars are 400 μm. Each value represents mean ± SD of three independent experiments. [a]Significantly different, compared with control siRNA-treated cells ($P < 0.05$ by ANOVA and Scheffé's test).

< 30%, compared with those in control siRNA-treated cells (Fig. 5C). When these cells were treated with RANKL for 5 days, numbers of TRAP-positive, multinucleated cells (Fig. 5D) and formation of resorption pits (Fig. 5E) were significantly reduced, compared with those of control siRNA-treated cells. In association with the impaired osteoclast formation, knockdown of Nox enzymes resulted in the reduction of RANKL-stimulated mRNA expression of NFATc1 as well as TRAP (Fig. 5F).

DISCUSSION

There are two crucial steps in osteoclast formation : commitment of progenitor cells to osteoclast precursor cells, and fusion of mononuclear cells to form TRAP-positive, multinucleated osteoclasts. The activation of the receptor for RANKL (RANK) is a key step in the differentiation of mature osteoclasts during the late stage (22-24). The RANK activation leads to the activation of distinct signaling cascades, such as nuclear factor (NF)-κB and mitogen-activated protein kinases (MAPKs), including p38 MAPK, c-Jun N-terminal kinases, and extracellular signal-regulated kinases, through several adaptor proteins and cofactors (25). RANKL also stimulates ROS generation, which has been suggested to be crucial for the activation of the RANK-signaling cascades leading to osteoclast differentiation (12). This is supported by the observations that addition of exogenous hydrogen peroxide stimulates osteoclastic bone resorption and cell motility (26). Alternatively, antioxidants can block the RANKL-dependent signaling (27, 28).

The most striking feature, observed during the RANKL-induced differentiation of RAW cells, was the marked reduction of Nox2 mRNA expression. Inversely related to this reduction, RANKL up-regulated Nox1 transcript expression. RANKL more slowly up-regulated Nox3 transcript expression and this roughly coincided with the appearance of TRAP-positive, multinucleated cells in RAW cells. In association with the marked down-regulation of Nox2 mRNA expression, the rate of spontaneous and PMA-stimulated $O_2^{\cdot-}$ production by RAW cell-derived osteoclasts decreased to about 8% and 3% of the respective control values, suggesting that the terminal differentiation of a monocyte-macrophage lineage cells into osteoclasts is associated with switching from a potent Nox2-based oxidase system in phagocytic cells to functionally different Nox systems that produce much smaller, but probably sufficient amounts of $O_2^{\cdot-}$ to change intracellular signals and functions. We also examined mRNA expression of the CCAAT displacement protein gene encoding a negative regulator for Nox2 gene transcription; however, RANKL did not change the mRNA level (data not shown). At present, the molecular mechanism for the Nox2 down-regulation is unknown. However, the abundant expression of Nox2 in osteoclasts, like in monocytes or macrophages, may be rather hazardous for bone health. Estrogen deprivation (29), homocysteine (28), or interferon γ(IFN-γ) (30) could activate osteoclast formation through increased ROS generation and NF-κB activation, which may contribute to osteoporosis by increasing bone resorption. Stimulation of osteoclast activity through IFN-γ involves Nox2 up-regulation (31). Thus, the decreased expression of Nox2 expression during osteoclast differentiation may be relevant to the maintenance of bone homeostasis.

RAW cells expressed $p22^{phox}$, two Nox organizer subunits ($p47^{phox}$ and NOXO1), one Nox activator ($p67^{phox}$), and two small GTPases (Rac1 and Rac2). RAW cells expressed a full set of components for Nox2 activity. According to the present knowledge on Nox1 enzymes and their regulatory proteins (7, 8), Nox1 can use $p67^{phox}$ instead of NOXA1 (9). Nox3 activity is facilitated by the presence of $p47^{phox}$ and $p67^{phox}$, NOXO1 and NOXA1, or NOXO1 alone (32). RAW cells did not express Nox4. Nox5 is not expressed in the mouse (7, 8). Thus, all Nox homologues detected in RAW cells are thought to become active enzymes. There are some difficulties in knockdown experiments. Induction of osteoclast differentiation with RANKL occurs at relatively low cell density; therefore, relatively higher concentrations of siRNA were necessary to keep their efficacy during 3-5 days of the differentiation period, which sometimes damaged cells and nonspecifically inhibited osteoclast formation. Therefore, to test whether ROS derived from any types of Nox were essential for osteoclast formation, we knocked down one target mRNA that was designed to totally block Nox-dependent ROS generation in RAW cells. Theoretically, it was expected that knockdown of $p22^{phox}$ would substantially remove all Nox enzyme activities, and that $p67^{phox}$ siRNA would profoundly eliminate the activities of Nox1, Nox2, and possibly Nox3. In fact, introduction of $p22^{phox}$ or $p67^{phox}$ siRNA significantly down-regulated $O_2^{\cdot-}$ to < 30% of the control level on day 3. These siRNAs partially, but significantly blocked osteoclast formation, which was

confirmed again by appearance of TRAP-positive, multinucleated cells, resorption pit formation, and expression of genes encoding a crucial transcription factor (NFATc1) and an osteoclast marker protein (TRAP). These results suggest that Nox-derived ROS may, at least in part, facilitate osteoclast differentiation.

The present study shows that RANKL stimulates switching between Nox homologues during osteoclast differentiation, and suggests that Nox-derived ROS may be essential for RANKL-induced osteoclast differentiation.

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