Free Radical Biology & Medicine 47 (2009) 189–199

Contents lists available at ScienceDirect

Free Radical Biology & Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

Receptor activator of nuclear factor-κB ligand-induced mouse osteoclast differentiation is associated with switching between NADPH oxidase homologues

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ARTICLE INFO

Article history:
Received 18 December 2008
Revised 6 April 2009
Accepted 23 April 2009
Available online 3 May 2009

Keywords:
Osteoclast differentiation
RANKL
NADPH oxidases
Reactive oxygen species
Free radicals

ABSTRACT

Reactive oxygen species (ROS) have been suggested to regulate receptor activator of nuclear factor-κB ligand (RANKL)-stimulated osteoclast differentiation. Stimulation of wild-type mouse bone marrow monocyte/macrophage lineage (BMM) cells by RANKL down-regulated NADPH oxidase 2 (Nox2) mRNA expression by half. RANKL reciprocally increased Nox1 mRNA levels and newly induced Nox4 transcript expression. BMM cells from Nox1 knockout ($Nox1^{-/-}$) as well as $Nox2^{-/-}$ mice generated ROS in response to RANKL and differentiated into osteoclasts in the same way as wild-type BMM cells, which was assessed by the appearance of tartrate-resistant acid phosphatase-positive, multinucleated cells having the ability to form resorption pits and by the expression of osteoclast marker genes. A small interfering RNA (siRNA) targeting Nox1 or Nox2 failed to inhibit the RANKL-stimulated ROS generation and osteoclast formation in wild-type cells, whereas Nox1 and Nox2 siRNAs significantly suppressed the ROS generation and osteoclast formation in $Nox2^{-/-}$ and $Nox1^{-/-}$ cells, respectively. We also confirmed that Nox4 siRNA did not affect the RANKL-dependent events in $Nox2^{-/-}$ cells, whereas $p22^{phox}$ siRNA suppressed the events in both wild-type and $Nox1^{-/-}$ cells. Collectively, our results suggest that there may be a flexible compensatory mechanism between Nox1 and Nox2 for RANKL-stimulated ROS generation to facilitate osteoclast differentiation.

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. In the presence of macrophage colony-stimulating factor (M-CSF), 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 characteristics 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 (Nox) consists of a membrane-integrated flavocytochrome b_{558}, composed of Nox2 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: the Nox and the dual oxidase families [7,8]. The Nox family comprises Nox1, Nox2 ($gp91^{phox}$), Nox3, Nox4, and Nox5. Recent knowledge of Nox enzymes and the actions of their regulatory proteins in forming active enzymes are summarized in recent reviews [7,8]. 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 controversial. Nox4 is also a $p22^{phox}$-dependent enzyme [10]. According to our present knowledge, Nox4 does not require cytosolic subunits [7,8]. 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 bones and secrete protons into an extracellular compartment between the osteoclast and the bone surface.

Abbreviations: M-CSF, macrophage colony-stimulating factor; ROS, reactive oxygen species; Nox, NADPH oxidase; RANKL, receptor activator of nuclear factor-κB ligand; BMM, mouse bone marrow monocyte/macrophage lineage; TRAP, tartrate-resistant acid phosphatase; $O_2^{\cdot-}$, superoxide anion; NOXO1, Nox organizer 1; NOXA1, Nox activator 1; CGD, chronic granulomatous disease; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; HBSS, Hanks' balanced salt solution; RT-PCR, reverse transcription polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NFATc1, nuclear factor of activated T cells, cytoplasmic 1; PMA, phorbol 12-myristate 13-acetate; SOD, superoxide dismutase; siRNA, small interfering RNA.

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doi:10.1016/j.freeradbiomed.2009.04.025

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 osteoclasts are suggested to participate in the complex process of bone resorption [2,4]. It has also been suggested that ROS 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^- [13], and no bone abnormalities have been documented in $Nox2^{-/-}$ mice or in patients with chronic granulomatous disease (CGD). These observations could be explained at least in part by the expression of Nox4 in mature osteoclasts [13,14] and Nox1 in osteoclast precursors [12]. In fact, Nox4 was reported to be responsible for O_2^- generation and resorption pit formation by osteoclasts [13,14]. A recent study has demonstrated that Nox1 is expressed in bone marrow monocyte/macrophage lineage (BMM) cells and suggested that Nox1 rather than Nox2 or Nox4 is important for RANKL-initiated production of ROS and differentiation into osteoclasts [12]. However, even in the case of $Nox1^{-/-}$ mice, no bone abnormalities have so far been reported [15,16]. These findings suggest the existence of an active compensatory mechanism for the ROS generation system in osteoclasts.

In this study, using BMM cells from wild-type, $Nox1^{-/-}$, and $Nox2^{-/-}$ mice, we examined the role of Nox homologues in RANKL-induced differentiation into osteoclasts and bone-resorbing activity.

Materials and methods

Reagents

Recombinant mouse RANKL was prepared as described previously [17]. Recombinant mouse M-CSF was obtained from R&D Systems (Minneapolis, MN, USA). 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, USA). 2′,7′-dichlorofluorescein diacetate (DCFH-DA) was from Invitrogen (Carlsbad, CA, USA). Submicrometer synthetic calcium phosphate thin-film-coated disks were purchased from BD Bioscience (Bedford, MA, USA). Dentine slices (200 μm [t]) were obtained from Wako Pure Chemical Co. (Osaka, Japan). The enhancer-containing luminol-based detection system (Diogenes) was obtained from National Diagnostics (Atlanta, GA, USA).

Mice

All mice were treated in accordance with the APS *Guiding Principles on the Care and Use of Animals,* and all procedures were approved by the Animal Care Committee of the University of Tokushima. C57BL/6J mice were purchased from the Shizuoka Laboratory Animal Center (Shizuoka, Japan). $Nox1^{-/-}$ mice were provided as previously described [15]. $Nox2^{-/-}$ mice were provided by Dr. Mary C. Dinauer, Indiana University School of Medicine (Bloomington, IN, USA) [18]. All mice were maintained under an artificial 12-h dark–light cycle (lights on at 8:30 AM) at a constant temperature of 23 ± 2°C and 65% humidity under specific-pathogen-free conditions. They were provided with a laboratory chow (Oriental Yeast, Tokyo, Japan) and water ad libitum.

Preparation and culture of BMM cells

After mice were sacrificed by decapitation under deep anesthesia with diethyl ether, bone marrow cells were isolated from tibiae and femurs of male C57BL/6J, $Nox1^{-/-}$, and $Nox2^{-/-}$ mice at 6 to 8 weeks of age according to the method described elsewhere [17,19]. In brief, after the isolated bone marrow cells were washed, red blood cells were removed using ammonium chloride buffer consisting of 150 mM ammonium chloride, 10 mM sodium bicarbonate, and 1 mM EDTA. Then, the bone marrow cells were cultured in phenol red-free α-minimum essential medium (α-MEM) containing 10% heat-inactivated fetal calf serum (FCS; Invitrogen), 100 U/ml penicillin G, and 100 μg/ml streptomycin at 37°C for 16 h under 95% air and 5% CO_2. Nonadherent cells were carefully layered onto a Ficoll (Histopaque-1077) density gradient solution and centrifuged at 400 g for 30 min at room temperature. BMM cells lying in the upper layer were harvested and washed. They were cultured in 24- ($0.5–1 \times 10^5$ cells/well), 48- ($2.5–5 \times 10^4$ cells/well), or 96-well (1×10^4 cells/well) culture plates with the same culture medium supplemented with 150 ng/ml RANKL and 100 ng/ml M-CSF. A half-volume of the RANKL-containing culture medium was replaced with the fresh medium every 3 days.

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 more than three nuclei were counted as osteoclasts under microscopic observation.

Bone-resorption assay

Bone-resorbing activity was assessed using submicrometer synthetic calcium phosphate thin-film-coated disks and dentine slices (200 μm [t]). BMM cells were cultured with 150 ng/ml RANKL and 100 ng/ml M-CSF for the indicated days on the calcium phosphate thin-film-coated disks in 24-well culture plates or on dentine slices in 96-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/). In the case of dentine slices, after being washed with 1 N NH_4OH for 15 min and then with water, the slice was stained with Mayer's hematoxylin solution for 1 min, and visualized resorption pits were photographed.

Quantitative real-time reverse transcriptase (RT)-PCR

Total RNA was extracted using an acid guanidium thiocyanate–phenol–chloroform mixture [20]. 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 RT-PCR. Specific primer sets for Nox1 (ABI Part 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), designed using the Primer Express program (Applied Biosystems, Foster City, CA, USA), were purchased from Applied Biosystems. cDNA was generated from 1 μg of total RNA with Multiscribe reverse transcriptase (Applied Biosystems) using oligo(dT)/hexamer primers. Quantitative real-time RT-PCR was performed using the ABI 7500 (Applied Biosystems). Data were normalized for the amount of GAPDH mRNA.