A crucial role for reactive oxygen species in macrophage colony-stimulating factor-induced RANK expression in osteoclastic differentiation

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Abstract. Macrophage colony-stimulating factor (M-CSF) is essential for differentiation from hematopoietic precursor cells into osteoclasts. M-CSF transiently increased the intracellular level of reactive oxygen species (ROS) through an NADPH oxidase (Nox) and induced the expression of receptor for activation of nuclear factor-κB (RANK) in early-stage osteoclast precursor cells (c-fms$^+$RANK$^-$). Blocking of the activity of Nox with diphenylene iodonium inhibited ROS production, activation of extracellular signal-regulated kinase (ERK), and the expression of RANK, PU.1 and MITF. The suppression of Nox2, but not Nox1, expression by RNA interference inhibited ROS production and RANK expression. These results suggested that ROS produced in response to M-CSF via a process mediated by Nox2 acted as an intracellular signaling mediator for RANK expression through the activation of ERK and the expression of PU.1 and MITF in early-stage osteoclast precursor cells.

Introduction

Reactive oxygen species (ROS) cause oxidative stress which has been implicated in the pathogenesis of various diseases. However, ROS have recently been recognized to play a role as a second messenger in various receptor signaling pathways (1-3). ROS were reported to regulate the survival or proliferation of monocyte/macrophages induced by macrophage colony-stimulating factor (M-CSF) (4-6). The role of ROS was also reported in osteoclast differentiation induced by receptor for activation of nuclear factor-κB ligand (RANKL) (7,8). However, little attention has been given to the role of ROS in M-CSF signaling at the early stage of osteoclastic differentiation.

Osteoclastic differentiation of hematopoietic progenitor cells requires M-CSF and RANKL (9) which act through their receptors c-Fms and receptor for activation of NF-κB (RANK), respectively. At the early stages of osteoclastic differentiation, M-CSF stimulates RANK expression in osteoclast precursor cells (c-fms$^+$RANK$^-$) and induces late-stage precursor cells (c-Fms$^+$RANK$^+$) which are common progenitors for macrophage/monocytes and osteoclasts (10). At later stages, the binding of RANKL to RANK induces commitment of c-Fms$^+$RANK$^+$ into mononuclear osteoclasts.

We previously reported that M-CSF generated ROS at the early stages of osteoclast differentiation in osteoclast precursor cells and that M-CSF induced RANK expression associated with the activation of extracellular signal-regulated kinase (ERK), but not p38 mitogen-activated protein kinase (MAPK) (11). In peripheral blood monocytes or bone marrow-derived monocyte/macrophages, M-CSF-generated ROS mediated the activation of ERK, p38 MAPK and Akt and the activation of Akt and p38 MAPK or Akt led to cell survival (5,6). Although these studies have reported the role of ROS in cell survival or proliferation in M-CSF signaling in monocyte/macrophages, the mechanisms by which M-CSF signaling leads to RANK expression remain poorly understood in early-stage osteoclast precursors.

The plasma membrane NADPH oxidase (Nox) is recognized as one of the major players in the generation of ROS (12). Aside from the neutrophil Nox2 isoform (13), other Nox isoforms have been discovered and the ROS they generate are suggested to act as messengers in the activation of specific signaling pathways (14). In bone marrow-derived hematopoietic stem cells, Nox1, Nox2 and Nox4 were reported to be expressed (15). The expression of Nox2 and lower levels of Nox1 and Nox1-mediated ROS generation by RANKL were reported in bone marrow-derived monocyte/macrophages (7). It was also suggested that a flexible compensatory mechanism existed between Nox1 and Nox2 for RANKL-stimulated

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Abbreviations: DCFH-DA, 2',7'-dichlorofluorescein diacetate; DPI, diphenyleneiodonium; ERK, extracellular signal-regulated kinase; JNK, Jun amino-terminal kinase; MAPK, mitogen-activated protein kinase; M-CSF, macrophage colony-stimulating factor; MNCs, multinucleated cells; Nox, NADPH (nicotinamide adenine dinucleotide phosphate) oxidase; RANK, receptor for activation of nuclear factor-κB; RANKL, receptor for activation of nuclear factor-κB ligand; ROS, reactive oxygen species; TRAP, tartrate-resistant acid phosphatase

Key words: reactive oxygen species, macrophage colony-stimulating factor, receptor for activation of nuclear factor κB, osteoclastic differentiation, nicotinamide adenine dinucleotide phosphate oxidase

ROS generation to facilitate osteoclast differentiation (16). However, the source of oxidant generated by M-CSF remained to be defined in early-stage osteoclast precursors.

In this study, we investigated the molecular basis for M-CSF-induced ROS generation and RANK expression in the early stages of osteoclastic differentiation. We found that the generation of ROS by M-CSF was mediated by Nox2 and was required for the expression of RANK associated with ERK activation and the expression of PU.1 and MITF.

Materials and methods

Preparation of the bone marrow osteoclast precursor cells. Bone marrow cells from the femur and tibia of 8-week-old Wistar/ST female rats were cultured for 16-24 h in MEM with 10% fetal calf serum (FCS) in the presence of M-CSF (5 ng/ml), and nonadherent cells were collected. The monocyte fraction at the interface after Ficoll-Paque gradient centrifugation of the nonadherent cells was used as bone marrow osteoclast precursor cells (17). Animals were treated in accordance with the protocols approved by the Animal Care Research Committee of Nara Women's University.

Determination of intracellular reactive oxygen species. Precursor cells were pre-cultured in the absence of M-CSF with or without diphenylene iodonium (DPI) or PD98059 for 30 min, and then stimulated with M-CSF (20 ng/ml). After the indicated time, cells were washed three times in MEM and incubated for 10 min with 2',7'-dichlorofluorescein diacetate (DCFH-DA) (10 μM). The fluorescence of DCF was detected by fluorescence microscopy as previously described (11). Fluorescence intensity was measured with WinRoof software (Mitani Co., Tokyo, Japan).

Determination of the activation of MAP kinase and Akt, and expression of c-fms, MITF, PU.1 and RANK. Precursor cells were pre-cultured in the absence of M-CSF with or without DPI or PD98059 for 30 min, and stimulated with M-CSF (20 ng/ml). Cells were harvested after stimulation for 5 min, and the phosphorylated protein levels of ERK, JNK, p38 and Akt were determined by western blot analysis. Following stimulation for 24 h, cells were used to determine the mRNA and protein levels of c-fms, PU.1, MITF and RANK by real-time RT-PCR and western blot analysis, respectively.

Quantitative real-time RT-PCR. Total RNA from the cell lysate was prepared using a commercial kit (Sepasol-RNA I Super G; Nacalai Tesque, Kyoto, Japan). The total RNA was reverse-transcribed with a first-strand cDNA synthesis kit (Toyobo, Osaka, Japan). Real-time PCR was performed using the cDNA, or total RNA for the negative control, with Thunderbird SYBR qPCR Mix (Toyobo) and specific primers (Table I) as previously described (18). Levels of gene expression were determined relative to an internal standard (actin) and expressed relative to the control values.

Western blot analysis. Equal amounts of protein of the cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to membranes. Western blotting and reprobing were performed and the chemiluminescent signals were quantified by a densitometer as reported (19). Antibodies recognizing actin (H-300), RANK (H-300), PU.1 (H-135), MITF (H-50) and p-ERK (E-4) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The antibodies to ERK, p38, pp38 (Thr180/Tyr182), JNK, pJNK (Thr183/Tyr185), Akt and pAkt (Ser473) were obtained from Cell Signaling Technology (Hitchin, UK). Protein concentrations were measured using the BCA protein assay kit (Pierce/Thermo Fisher Scientific Inc., Rockford, IL, USA).

Osteoclastic differentiation of bone marrow osteoclast precursor cells. Precursor cells ($1x10^4$ cells/well of a 96-well plate or $1.5x10^5$ cells/35-mm plate) were cultured in MEM with 10% FCS containing M-CSF (20 ng/ml) and RANKL (10 ng/ml) with or without DPI or PD98059. Cultures were maintained with a change of medium every 3 days. After 5 days, cells were used for counting the tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells (MNCs) after TRAP staining and the assessment of cell viability with a leukocyte acid phosphatase kit 387-A (Sigma) and WST-8 (Cell Counting kit-8; Dojin, Japan), respectively, as previously described (20).

RNA interference. The duplexed Stealth™ siRNA designed against Nox1 and Nox2 (Cybb), and the negative control were purchased from Invitrogen. The sequences were: Nox1 siRNA, 5'-CCAAGGUUGUCAUGCACCCAUGUAA-3' and 5'-UUACAUGGGUGCAUGACAACCUUGG-3'; Nox2 (Cybb) siRNA, 5'-GAUUCAGGAUGGAGGUGGGACAA UA-3' and 5'-UAUUGUCCCACCUCCAUCCUGAAUC-3'. Precursor cells were seeded at a density of $4x10^5$ cells/dish in 35-mm-diameter culture dishes or $2x10^5$ cells/well in 96-well culture plates and transfected with 25 nM of negative control siRNA (Stealth RNAi™ Negative Control; Invitrogen), Nox1 siRNA, or Nox2 siRNA using X-tremeGENE HP DNA Transfection Reagent (Roche Applied Science, Penzberg, Germany) for 24 h, according to the manufacturer's instructions. After a change to fresh medium, the expression of Nox1 and Nox2 was determined by RT-PCR. ROS production and RANK expression were determined at 5 min and 24 h after stimulation with M-CSF (20 ng/ml), respectively.

Statistical analysis. All statistical analyses were performed using Welch's method with the Microsoft Excel data analysis program. The differences were considered statistically significant at $P<0.05$. All data are expressed as the mean ± SEM.

Results

M-CSF generates ROS in osteoclast precursor cells. Stimulation of osteoclast precursor cells with M-CSF resulted in an increase in the intensity of DCF fluorescence (Fig. 1A), indicating that M-CSF induced intracellular ROS production. The production of ROS rapidly increased to a maximum level at approximately 5 min after the M-CSF treatment and thereafter decreased toward the basal level (Fig. 1).

Effects of NADPH oxidase inhibitor and ERK inhibitor on M-CSF-induced ROS production, MAP kinase and Akt activa-

Table I. Primers used for quantitative real-time PCR.

Target	Forward primer sequence	Reverse primer sequence
Actin	AGCCATGTACGTAGCCATCCA	TCTCCGGAGTCCATCACAATG
c-fms	TAGAGCCAGGTGCAACAGTG	CGCATAGGGTCTTCAAGCTC
MITF	TTGGAAGACATCCTGATGGAC	GCTGCTTGTTTTCGAAGCTC
Nox1	CCCTTTGCTTCCTTCTTGAAATC	GCACCCGTCTCTCTACAAATCC
Nox2	TGATCATCACATCCTCCACCAA	GATGGCAAGGCCGATGAA
Nox3	GCAGCATTGGCGTGTTCTT	GAAATGAACGCCCCTAGGATCT
Nox4	CTGCATCTGTCCTGAACCTCAA	TCTCCTGCTAGGGACCTTCTGT
PU.1	TGGAGAAGCTGATGGCTTG	CCTTGTGCTTGGACGAGAA
RANK	ATATGCCTGCATCCCCTGAA	TAGCCATCCGTTGAGTTGGA

Nox, nicotinamide adenine dinucleotide phosphate oxidase; RANK, receptor for activation of nuclear factor-κB.

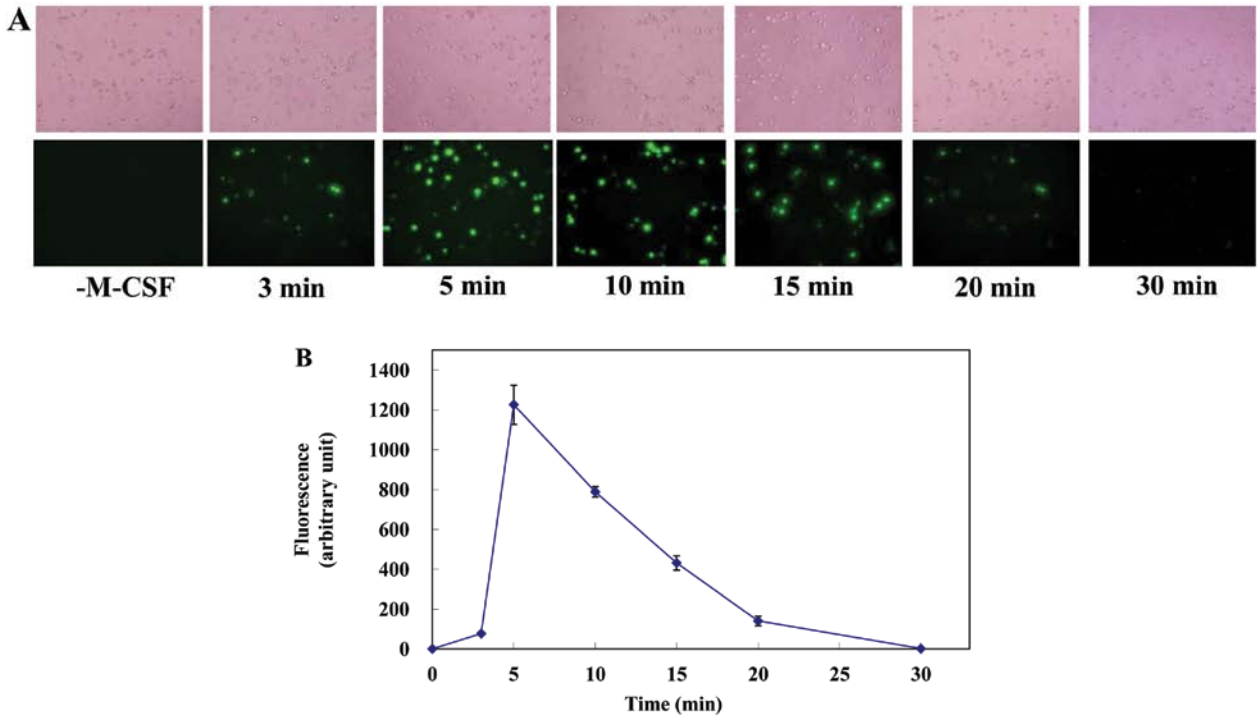

Figure 1. M-CSF-induced ROS production. Precursor cells were treated with M-CSF (20 ng/ml) for the period indicated. After the addition of DCFH-DA, the fluorescence of DCF was detected. (A) Representative microscopic fields (x400 magnification). (B) DCF fluorescence. Values are the mean ± SEM of four experiments.

tion, mRNA and protein levels of PU.1, MITF and RANK, and osteoclast formation. Treatment of osteoclast precursor cells with DPI, a specific inhibitor for flavoprotein that is a constituent of the Nox complex, eliminated the rise in DCF fluorescence induced by M-CSF (Fig. 2A). The ERK inhibitor, PD98059, did not affect the production of ROS. ERK and JNK were activated by M-CSF, but the phosphorylation of p38 was not detected (Fig. 2B). DPI treatment blocked the activation of ERK but not JNK. The phosphorylated Akt was also increased by M-CSF, but this activation was not inhibited by DPI or PD98059 (Fig. 2B).

M-CSF increased the mRNA levels of RANK, PU.1 and MITF to ~10-, 2.4- and 2.8-fold the uninduced (before the stimulation of M-CSF) value, respectively, although the c-fms mRNA levels were unchanged (Fig. 2C). DPI or PD98059 significantly suppressed these increases. The protein levels of RANK, PU.1 and MITF were also increased by M-CSF and these increases were suppressed by DPI or PD98059 (Fig. 2B). The osteoclastic differentiation of precursor cells was significantly inhibited by the presence of DPI or PD98059 without affecting cell viability (Fig. 2D).

Expression of Nox isozyme. Precursor cells expressed the mRNA of Nox2, a smaller amount of Nox1, and undetectable levels of Nox3 and Nox4. The mRNA levels of Nox1 were ~0.1% of these of Nox2. M-CSF decreased Nox2 expression to ~30% of the uninduced level, but increased the Nox1 expression to 4-fold the uninduced level (Fig. 3). The levels of Nox3 and Nox4 were undetectable after the induction by M-CSF.

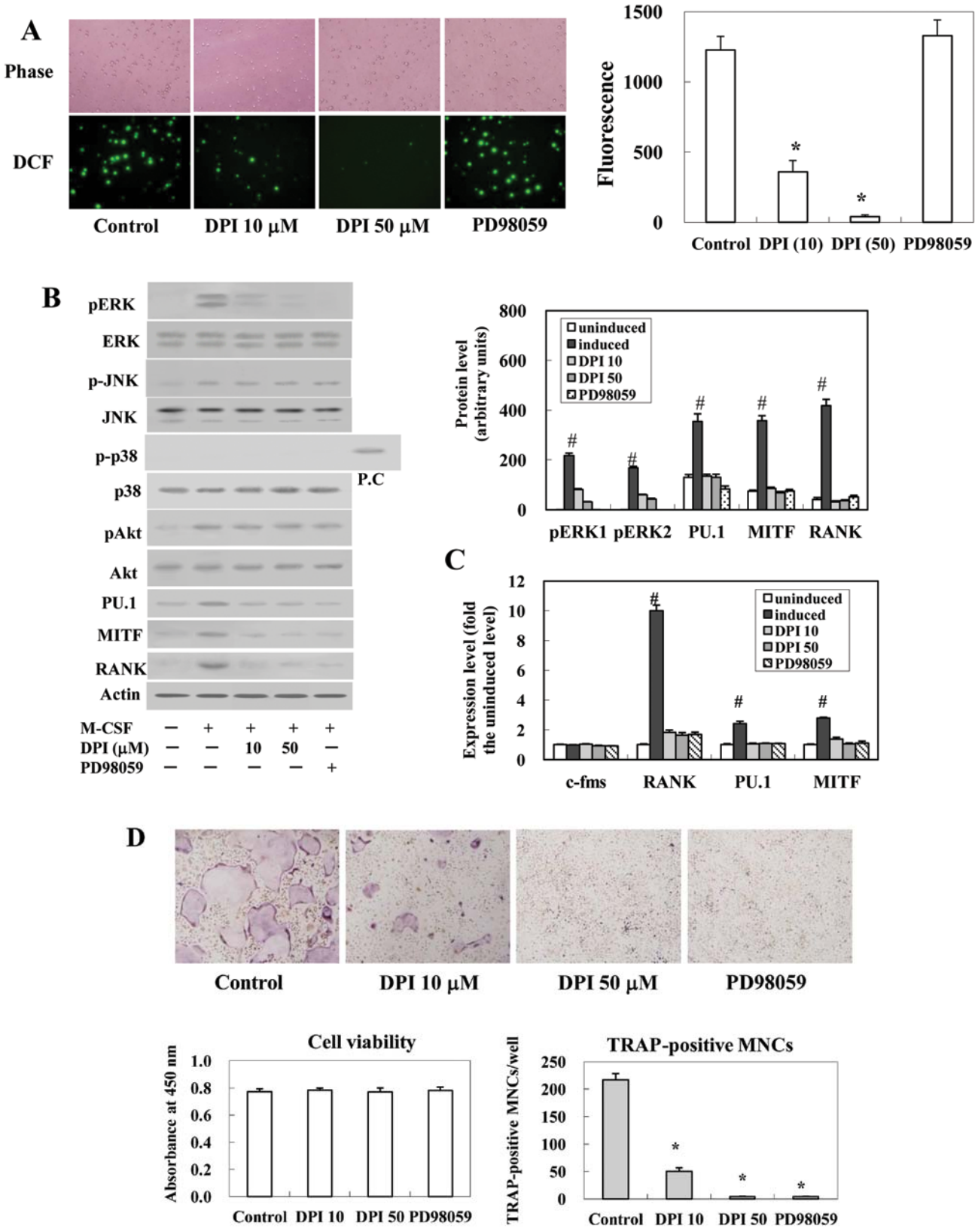

Figure 2. Effects of DPI and PD98059 on M-CSF-induced ROS production, the activation of MAP kinase and Akt, the mRNA and protein levels of c-fms, MITF, PU.1 and RANK, and osteoclastogenesis. Precursor cells were pre-cultured in the absence of M-CSF with or without DPI or PD98059 for 30 min and then stimulated with M-CSF (20 ng/ml). (A) After stimulation for 5 min, DCFH-DA was added and the fluorescence of DCF was detected. Representative microscopic fields (x400 magnification; left panel) and quantitative calculation data (right panel) are shown. (B) The activation of ERK, JNK, p38 MAPK and Akt of the cells after stimulation for 5 min, and the protein levels of PU.1, MITF and RANK after the stimulation for 24 h were determined by western blotting. The protein levels of phosphorylated ERK1 and 2 (pERK1, pERK2), PU.1, MITF and RANK were quantified by densitometry and are graphically represented (right panel). (C) After stimulation for 24 h, cells were harvested and the expression levels of c-fms, MITF, PU.1 and RANK were determined by real-time RT-PCR. Levels are expressed relative to the uninduced level. (D) Precursor cells were cultured with M-CSF (20 ng/ml) and RANKL (10 ng/ml) for 5 days. Cell viability and the number of TRAP-positive MNCs were determined. Values are the mean ± SEM of four experiments. Significantly different from the control value ($^{*}P<0.05$). Significantly different from the uninduced value ($^{\#}P<0.05$).

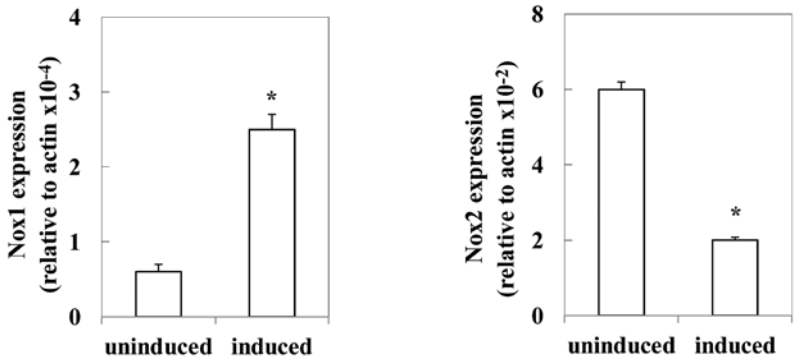

Figure 3. Expression of Nox. The mRNA levels of Nox1 and Nox2 in precursor cells before (uninduced) or at 24 h after the stimulation with M-CSF (20 ng/ml) (induced) were determined by real-time RT-PCR. Levels are expressed relative to the actin level. Values are the mean ± SEM of four experiments. Significantly different from the uninduced value ($^{*}P<0.05$).

Effects of Nox1 and Nox2 siRNA on M-CSF-induced ROS generation and RANK expression. To investigate which Nox isozyme is responsible for the responses to M-CSF, precursor cells were treated with negative control, Nox1 or Nox2 siRNA, and ROS production and RANK expression were examined (Fig. 4). Nox1 or Nox2 was effectively knocked down by the specific siRNA, as shown by the real-time RT-PCR analysis (Fig. 4A). The silencing of Nox2 in precursor cells resulted in a significant decrease in ROS production in response to M-CSF (Fig. 4B). However, Nox1 knockdown had no effect on ROS production. Nox2, but not Nox1, siRNA inhibited the expression of RANK (Fig. 4C). These results suggest that Nox2 is a critical mediator of M-CSF-induced RANK expression in precursor cells (c-fms^{+}RANK^{-}).

Discussion

There are two types of osteoclast precursor cells, the early-stage precursor cells expressing c-Fms, but not RANK (c-fms^{+}RANK^{-}), and the late-stage precursor cells expressing c-Fms and RANK (c-Fms^{+}RANK^{+}) (10). M-CSF stimulated RANK expression in early-stage osteoclast precursors (c-fms^{+}RANK^{-}) and the binding of RANKL to RANK triggers the differentiation of late-stage precursors into osteoclasts. The expression of RANK caused by the binding of M-CSF is a key step in the early stages of osteoclastogenesis.

This study clearly demonstrated a critical role for ROS in the differentiation of early-stage osteoclast precursor cells into late-stage precursors. M-CSF generated ROS in the early-stage of osteoclast precursor cells. The production of ROS was inhibited by a Nox inhibitor, DPI, indicating Nox-mediated ROS generation. The inhibition of ROS production resulted in the suppression of RANK expression. This study, for the first time, revealed that Nox-mediated production of ROS was required for the expression of RANK in early-stage osteoclast precursor cells. In agreement with our previous finding (20), M-CSF activated ERK and JNK, but not p38. The M-CSF-activation of ERK, but not JNK, was inhibited by DPI treatment. Consistent with these results, the expression of RANK was inhibited by DPI or a specific inhibitor of ERK, PD98059. The mRNA and protein levels of PU.1 and MITF, which transactivate RANK expression (21), were also reduced by DPI or PD98059. Furthermore, PD98059 did not inhibit ROS production, but suppressed RANK expression. These results suggested that ERK activation functioned downstream of ROS-generation and upstream of RANK expression in M-CSF signaling in early-stage osteoclast precursors. The pathway, M-CSF/ROS generation/ERK activation/RANK expression, was suggested to occur in the early stages of osteoclastogenesis. In peripheral blood monocytes, the activation of ERK by M-CSF was suggested to play a role in cellular survival (4). Macrophages from p47$^{phox-/-}$ mice, lacking a key component of the Nox complex required for ROS generation, had no effect on M-CSF-stimulated ERK expression, but reduced cell survival and Akt1 and p38 phosphorylation (5). Application of DPI was reported to inhibit the responses of monocyte/macrophages to M-CSF, including ROS production, cell proliferation, and phosphorylation of c-Fms and Akt kinase, but not MAP kinases such as ERK, p38 and JNK (6). These studies reported that Nox-mediated ROS generation by M-CSF led to the activation of p38 and Akt and survival in monocyte/macrophages. However, in our experiments using early-stage osteoclast precursor cells, neither the activation of p38 by M-CSF nor the inhibition of Akt activation was observed with the Nox inhibitor, DPI. DPI did not reduce the viability of cultured precursor cells, although it decreased osteoclast formation. These results suggested that the activation of Akt by M-CSF was not mediated by Nox-generated ROS in early-stage osteoclast precursor cells different from the differentiated monocyte/macrophages and was not involved in the signaling pathway for M-CSF-induced RANK expression. The M-CSF signaling may differ depending on the stage of cell differentiation, although osteoclast precursors can differentiate into monocyte/macrophages.

Nox is recognized as a major intracellular source of ROS. As observed in monocyte/macrophages (7,16), Nox2 was found to be the main isotype expressed in early-stage osteoclast precursor cells. The expression of Nox1 was also detectable at a low level, whereas that of other members such as Nox3 and Nox4 was undetectable. M-CSF decreased the expression of Nox2 and increased that of Nox1. The induced level of Nox1 expression was still 1% of the induced level of Nox2. The downregulation of Nox2 and the upregulation of Nox1 were also observed on RANKL-stimulation (8,16). Although the upregulation of Nox4 by RANKL-stimulation was also reported (16), the increase in its expression by M-CSF was not

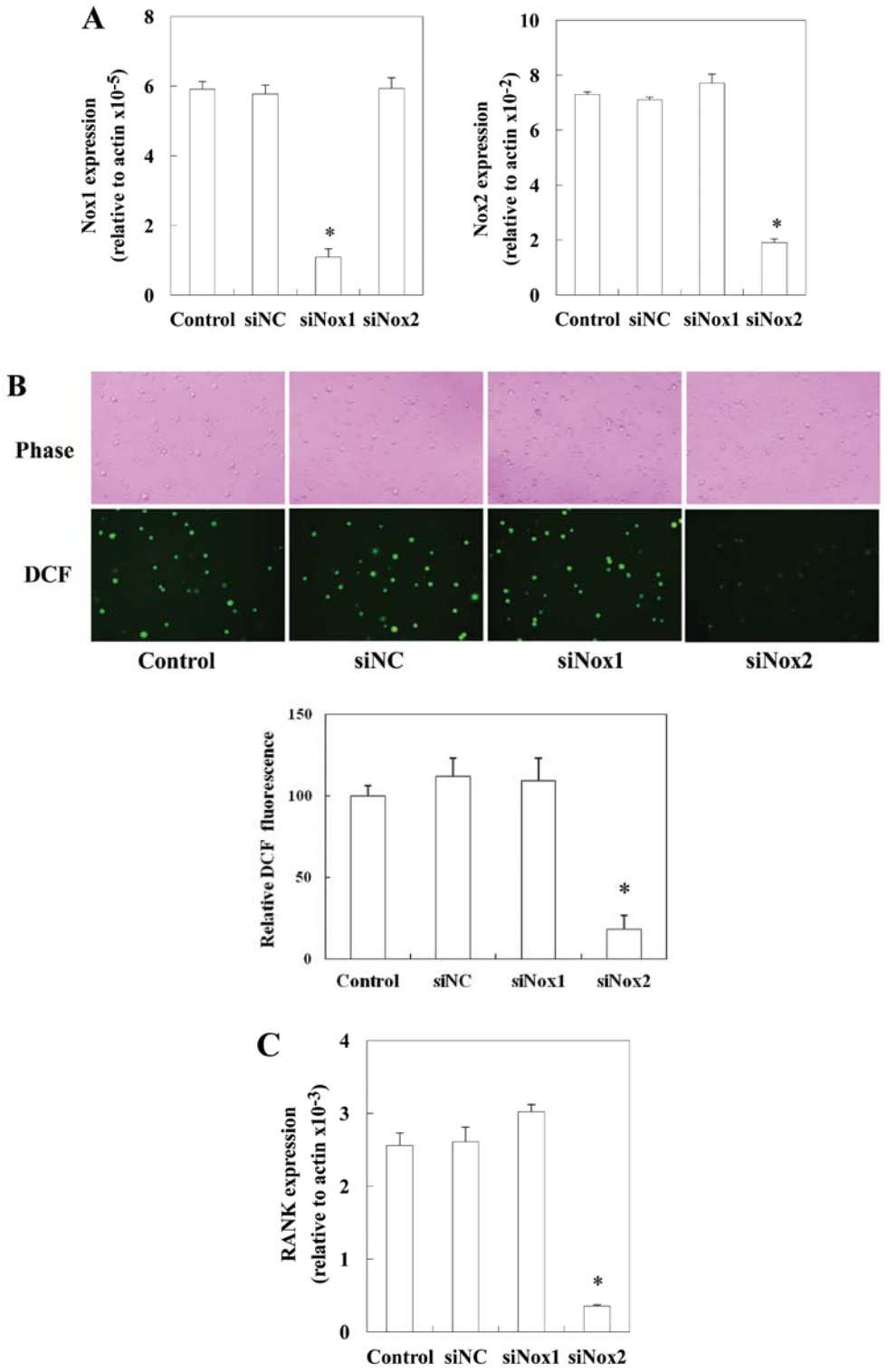

Figure 4. Effects of negative control, Nox1 and Nox2 siRNA on M-CSF-induced ROS generation and RANK expression. Precursor cells were transfected with or without negative control siRNA (siNC), Nox1 siRNA (siNox1), or Nox2 siRNA (siNox2) using transfection reagent for 24 h. (A) The expression of Nox1 and Nox2 was determined by real-time RT-PCR. Expression levels are expressed relative to the actin level. Values are the mean ± SEM of three experiments. Significantly different from the control value (*P<0.05). (B) After treatment with M-CSF (20 ng/ml) for 5 min, ROS production was determined. Representative microscopic fields are shown (x400 magnification; upper panel). DCF fluorescence was expressed relative to the control value (lower panel). Values are the mean ± SEM of three experiments. Significantly different from the control value (*P<0.05). (C) After treatment with M-CSF (20 ng/ml) for 24 h, RANK expression was determined by real-time RT-PCR. Expression levels are expressed relative to the actin level. Values are the mean ± SEM of three experiments. Significantly different from the control value (*P<0.05).

observed in this study. The siRNA targeting Nox2, but not Nox1, inhibited the M-CSF-stimulated ROS production and RANK expression. These results clearly indicated the generation of ROS by M-CSF to be mediated through Nox2 in early-stage osteoclast precursors, although a flexible compensatory mechanism between Nox1 and Nox2 for RANKL-stimulated

ROS production was suggested in the osteoclast differentiation of marrow-derived monocyte/macrophages (16).

In conclusion, this study provides evidence that ROS produced in response to M-CSF via a process mediated by Nox2 act as an intracellular signaling mediator for RANK expression through the activation of ERK and the expression of PU.1 and MITF in early-stage osteoclast precursor cells (c-fms$^+$RANK$^-$).

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