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Protein phosphatase 2A Cα is involved in osteoclastogenesis by regulating RANKL and OPG expression in osteoblasts

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ABSTRACT

We examined whether alteration of PP2A Cα expression in osteoblasts is involved in osteoclast differentiation. Reduction of PP2A Cα in MC3T3-E1 cells (shPP2A) decreased receptor activator of nuclear factor κB ligand (RANKL) expression and increased osteoprotegerin (OPG) expression. The conditioned medium from shPP2A cells failed to induce NFATc1 as well as the expression of osteoclast marker genes cathepsin K and osteoclast-associated receptor (OSCAR) in bone marrow macrophage cells. Treatment of bone marrow macrophage cells with the conditioned medium from shPP2A cells impaired osteoclastogenesis. These results suggest that alteration of PP2A Cα expression in osteoblasts modulates the expressions of RANKL and OPG, which are involved in osteoclastogenesis via the NFATc1 transcription factor.

1. Introduction

The bone is a dynamic tissue that provides mechanical support, physical protection, and a storage site for systemic mineral homeostasis. Bone remodeling is carried out by osteoblasts and osteoclasts derived from independent lineages (mesenchymal and hematopoietic). Osteoblasts produce both stimulatory and inhibitory factors that tightly regulate osteoclast formation and activity [1–3].

A member of the tumor necrosis factor (TNF) family—receptor activator of nuclear factor κB ligand (RANKL)—has been shown to regulate osteoclast differentiation and function directly [4,5]. RANKL is found both as a transmembrane molecule on osteoblasts and as a secreted molecule—soluble RANKL (sRANKL) [6]. Binding of RANKL to its receptor, RANK, on the surface of osteoclast precursor cells initiates signals, which leads to osteoclastogenesis. On the other hand, osteoprotegerin (OPG) is a competitive receptor for RANKL and has been shown to prevent bone destruction by blocking the binding of RANKL with the receptor RANK, thereby inhibiting osteoclast differentiation and activation [4,7]. Therefore, alterations in the ratio of RANKL/sRANKL to OPG produced from osteoblasts affect bone turnover and homeostasis by controlling osteoclastogenesis [8,9].

The binding of RANKL/sRANKL to its receptor RANK on the surface of osteoclast precursor cells activates various transcription factors, including nuclear factor of activated T cells c1 (NFATc1) [10–12]. In response to an increase in intracellular Ca^{2+} levels, NFATc1 is dephosphorylated, which allows NFATc1 to translocate from the cytoplasm to the nucleus [10,13]. Translocated active NFATc1 cooperates with other transcription factors to regulate the expression of many osteoclast-specific genes such as cathepsin K, osteoclast-associated receptor (OSCAR), and tartrate-resistant acid phosphatase (TRAP) [3,14–16].

We recently reported that the reduction of PP2A Cα accelerates bone formation and osteoblast differentiation through the expression of bone-related genes [17]. However, it remains unknown whether reduction of PP2A Cα expression in osteoblasts has an effect on osteoclast differentiation. The purpose of this study was to examine whether PP2A Cα expression in osteoblasts is involved in osteoclast differentiation through RANKL/OPG expression and the NFATc1 transcription factor.

2. Materials and methods

2.1. Materials

Alpha-modified Eagle's minimal essential medium (α-MEM) was purchased from Invitrogen (Carlsbad, CA). Plastic dishes were obtained from IWAKI (Chiba, Japan) and fetal bovine serum (FBS)

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was purchased from JRH Biosciences (Lenexa, KS). Antibodies against RANKL (sc-9037) and OPG (sc-8468) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), while anti-Gapdh antibody, ascorbic acid, β-glycerophosphate, Fast Red TR, and naphthol AS-MX phosphate were all purchased from Sigma-Aldrich (St. Louis, MO). The other materials used were of the highest grade commercially available.

2.2. Cell culture and differentiation

PP2A knockdown (shPP2A) and control (shCont) osteoblastic cells used in this study have been described previously [17]. Mouse macrophage RAW264.7 cells were obtained from Riken Cell Bank (Tsukuba, Japan). shPP2A and shCont cells were cultured in α-MEM supplemented with 10% fetal bovine serum (FBS) at 37 °C under a humidified atmosphere of 5% CO_2. For each assay, the cells were plated onto 100-mm culture dishes and cultured with differentiation medium (supplemented with 50 μM ascorbic acid and 2 mM β-glycerophosphate) for 7 days. The conditioned medium was harvested, centrifuged to remove cell debris, filtered through a 0.45-μm pore membrane filter (ADVANTEC, Tokyo, Japan), and stored at −80 °C.

2.3. Real-time PCR analysis

Total RNA was extracted using Trizol, as recommended by the manufacturer (Invitrogen). Real-time PCR of each gene was performed in triplicate for at least three independent experiments. Real-time PCR was performed with a 7300 Real-time PCR system (Applied Bio-systems, Carlsbad, CA) using SYBR Premix Ex Taq™ (Takara Bio, Kyoto, Japan). The sequences of the primers were as follows: Mouse Gapdh (NM_008084): (forward, 761) 5′-TGTGTCCGTCGTCGTGGATCTGA-3′, (reverse, 910) 5′-TTGCTGTTGAAGTCGCAGGAG-3′; Mouse cathepsin K (NM_007802): (forward, 620) 5′-CAGCAGAACGGAGGCATTGA-3′, (reverse, 703) 5′-CTTTGCCGTGGCGTTATACATACA-3′; Mouse OSCAR (NM_175632): (forward, 116) 5′-TGCATGCCGTGCTGACTTC-3′, (reverse, 223) 5′-AAGGTCACGTTGATCCCAGGAG-3′.

2.4. ELISA

ELISA was performed according to the method described by Hirota et al. [18], with some modifications. Briefly, 50 μl of the conditioned media each from shCont and shPP2A cells were separately dispensed into a polystyrene ELISA plate (Nunc Immuno Module, Maxisorptype, Nalge Nunc, Rochester, NY). Subsequently, the plates were dried completely at 37 °C. After blocking with 1.5% bovine serum albumin in PBS for 1 h at room temperature (RT), these plates were incubated with 50-μl total volume of 1:100 diluted antibody for RANKL or OPG at RT for 1 h. Normal rabbit or goat IgG was used as a negative control. After washing three times with PBS, the plate was incubated with 1:500 diluted horseradish peroxidase-labeled anti-rabbit (for RANKL) or -goat (for OPG) (Biosource Co., Camarillo, CA) secondary antibodies for 1 h at RT. The plate was washed 3 times with PBS and then incubated with peroxidase substrate, 2,2′-azino-bis-(3-ethyl-benzthiazolin sulfonate) at RT for 15 min. The reaction was measured spectrophotometrically at 405 nm.

2.5. Bone marrow cells

All mice studied were reared in our specific pathogen-free mouse colony and given food and water ad libitum. Experiments were humanely conducted under the regulation and permission of the Animal Care and Use Committee of the University of Tokushima, Tokushima, Japan (toku-dobutsu 10051). Bone marrow-derived cells from Balb/c mice (4–6 weeks of age) were incubated with the conditioned medium from shCont and shPP2A cells. The cultured cells were fixed and stained with TRAP staining solution. TRAP-positive multinuclear cells containing more than three nuclei were counted as osteoclasts.

2.6. Immunocytochemistry

Bone marrow macrophage cells were grown on sterile 18-mm round glass cover slips placed in 60-mm plastic dishes. The cover slips were incubated for 30 min with an anti-NFATc1 antibody (BD Biosciences, San Jose, CA) diluted to 1:200 in the blocking solution. Then, they were incubated for 30 min with Alexa Fluor 488 goat anti-mouse IgG (Invitrogen) secondary antibody, diluted to 1:500 in 4% BSA. After incubation with 10 μg/ml Hoechst 33342 for 30 min, the cover slips were mounted with PermaFluor aqueous mounting medium (Lipshow, Pittsburgh, PA).

2.7. Statistical analysis

All data was expressed as mean ± SD, and a minimum of three independent experiments were performed for each assay. Analysis of variance (ANOVA) was used for statistical analysis. Statistical significance was indicated with "*", and P values less than 0.01 were considered significant.

3. Results

3.1. Reduction of PP2A Cα attenuates RANKL expression in osteoblasts

To examine whether PP2A Cα expression plays a role in the expressions of RANKL and OPG in osteoblasts, mRNA isolated from shCont and shPP2A cells were subjected to real-time PCR using specific primers for these transcripts (Fig. 1A). The level of RANKL was found to be significantly lower in shPP2A cells compared to shCont cells, whereas OPG expression was significantly higher in shPP2A cells compared to shCont cells. Consistent with the real-time PCR data, the ratio of sRANKL/OPG in the cultured medium of shPP2A cells was lower than that of shCont cells, as determined using ELISA (Fig. 1B).

3.2. Conditioned medium of shPP2A cells does not induce NFATc1 translocation

During osteoclastogenesis, an activated form of NFATc1 is known to translocate from the cytoplasm into the nucleus in osteoclast precursor cells. Therefore, we investigated whether the conditioned medium from shCont and shPP2A cells could stimulate NFATc1 translocation. Following a 1 h treatment of bone marrow macrophage cells with either conditioned medium, the cells were fixed, permeabilized, and stained using anti-NFATc1 antibody and Hoechst 33342. We observed green fluorescent signal mostly in the cytoplasm indicating that NFATc1 expression was localized to the cytoplasm in untreated cells (Fig. 2, upper panel). When we treated bone marrow macrophage cells with the conditioned medium from shCont cells, intense green fluorescence for NFATc1 was observed in the nucleus (Fig. 2, middle panel). In contrast, strong fluorescence was not observed in the nucleus of the cells treated with the conditioned medium from shPP2A cells (Fig. 2, bottom panel). Nuclear staining with Hoechst 33342 was visualized as a red pseudo-color. Thus, from a merged image, we observed a yellow fluorescent signal restricted to the nucleus of cells treated with the conditioned medium from shCont, but not shPP2A cells, thereby confirming NFATc1 localization in the nucleus of these cells. Same results were obtained from another experiment, in which we trea-