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MicroRNA-183 increases osteoclastogenesis by repressing heme oxygenase-1

Ke Ke [1], Ok-Joo Sul [1], Monisha Rajasekaran, Hye-Seon Choi *

Department of Biological Sciences (BK21 Program), University of Ulsan, Ulsan 680-749, South Korea

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ABSTRACT

Emerging evidence suggests that microRNAs (miRs) influence skeletal structure by modulating osteoclastogenesis and bone resorption. We have demonstrated previously that the up-regulation of heme oxygenase-1 (HO-1) attenuated osteoclastogenesis in bone marrow-derived macrophages (BMMs). RANKL-induced osteoclastogenesis elevates microRNA-183 (miR-183) in BMM. We show here that HO-1 is a target gene of miR-183 and that this miRNA binds to the 3′-UTR of HO-1. We find that a synthetic inhibitor that binds to miR-183 decreases osteoclast (OC) differentiation and increases the expression of HO-1, while a mimic of endogenous mature miR-183 has the opposite effect. Moreover, the HO-1 inducers, resveratrol and piceatannol decrease the expression of miR-183, resulting in attenuated osteoclastogenesis. Our findings reveal how miR-183 affects OC formation.

1. Introduction

MicroRNAs (miRs) are 21–23 nucleotide non-coding RNA molecules that regulate the stability and the translation of target mRNA. Each miR is transcribed as a primary miR (pri-miR) containing a 5′-cap structure and poly (A) tail, from which is generated the mature miR [1]. Conversion to the mature miR requires two nuclear enzymes, the nuclear RNaseIII, Drosha, and the cytosolic RNaseIII, Dicer, which act sequentially. More than 700 different miRs have been found in the mouse genome (miRBase http://microrna.sanger.ac.uk). Generally each miR acts on multiple target mRNA, such that about one-third of protein-coding genes are modulated by miRs. As a result, miRs are one of the largest classes of gene-regulatory molecules, and they affect cell differentiation, proliferation, cell death, metabolic control, and most other biological processes. Mutations of miR-2861 are associated with primary osteoporosis in humans [2] and overexpression of miR-34c induces age-related osteoporosis in mice [3], suggesting that miRs play important roles in bone disease. In addition, inactivation of the myeloid-specific Dicer induces osteopetrosis due to decreased differentiation and activity of osteoclasts (OCs), implying that osteoclastogenesis requires miR expression [4].

OC formation occurs when bone marrow macrophages (BMMs) derived from hematopoietic stem cells are stimulated by two essential osteoclastogenic factors: macrophage-colony stimulating factor (M-CSF) and receptor activator of nuclear factor-κB ligand (RANKL) [5]. RANKL drives the main process of differentiation, while M-CSF plays a role in proliferation and survival during differentiation. Binding of RANKL to its receptor, RANK, induces and activates the transcription factors nuclear factor-κB (NF-κB), c-Fos, and nuclear factor of activated T cells, cytoplasmic 1 (or NFAT2), resulting in expression of key osteoclastogenic genes such as tartrate-resistant acid phosphatase (TRAP), cathepsin K, calcitonin receptor, DC-STAMP, and ATP6v0d2 [6]. Increased OC formation is responsible for excess bone resorption, leading to the bone loss seen in osteoporosis, and inflammation-induced bone destruction.

Reactive oxygen species (ROS) have long been implicated in stimulating OC differentiation [7], and oxidative stress is thought to contribute to the increased bone resorption leading to osteoporosis [8]. The induction of antioxidants such as heme oxygenase-1 (HO-1) is thought to counteract the deleterious effect of oxidative stress. HO-1 is the rate-limiting enzyme in heme catabolism, cleaving heme to generate biliverdin, carbon monoxide, and iron. HO-1-deficient mice suffer from chronic inflammation that progresses with age [9] and HO-1 has been shown to have cyto-protective effects in various cell types [10,11]. These findings suggest that targeted induction of this stress-response enzyme might be a useful strategy for protecting against inflammatory processes and oxidative tissue damage. Resveratrol (trans-3,4,5-trihydroxystilbene) is a natural polyphenolic stilbene, found in grapes and various other fruits [12], which inhibits OC formation by impairing NF-kB activation [10]. Resveratrol-mediated HO-1 induction has been reported to have neuroprotective effects in neuronal cultures [11]. Piceatannol, a tetrahydroxystilbene analog of resveratrol, is made by the plant, *Euphorbia lagascae* [13] and inhibits the Syk nonreceptor

Abbreviations: BMM, bone marrow macrophages; HO-1, heme oxygenase-1; M-CSF, macrophage-colony stimulating factor; miR, microRNA; miR-183, microRNA-183; MNC, multinucleated cells; NFAT2, nuclear factor of activated T cells, cytoplasmic 1; NF-κB, nuclear factor-κB; OC, osteoclast; ROS, reactive oxygen species; RANKL, receptor activator of nuclear factor-κB ligand; TRAP, tartrate-resistant acid phosphatase.

* Corresponding author at: Department of Biological Sciences, University of Ulsan, Ulsan 680-749, South Korea.
E-mail address: hschoi@mail.ulsan.ac.kr (H.-S. Choi).

[1] The first two authors contributed equally to this work.

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kinase [14] and NF-kB activation [15]. It decreases LPS-induced inflammation by inducing HO-1 [16]. However, the role of HO-1 is highly debatable, since some contradictory effects have been demonstrated [9–11,17], suggesting that there may be unidentified regulatory factors involved in HO-1 functions.

Previously we found that RANKL decreased the expression of HO-1 and up-regulation of HO-1 decreased osteoclastogenesis [18,19]. In this study, we investigated whether the expression of HO-1 was associated with specific miR during RANKL-induced osteoclastogenesis in OC. We explored the idea that the expression of miR-183 modulated RANKL-induced OC differentiation by targeting HO-1. The HO-1 inducers, piceatannol and resveratrol reduced miR-183 which resulted in increased HO-1, and led to impaired osteoclastogenesis.

2. Materials and methods

2.1. Reagents and antibodies

Recombinant mouse M-CSF and RANKL were obtained from R&D systems, Inc. (Minneapolis, MN). Piceatannol and resveratrol were obtained from Sigma Chemical (St. Louis, MN) and dissolved as stock solutions in dimethyl sulfoxide.

2.2. Cells and OC formation

Raw264.7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (GIBCO BRL, Grand Island, NY, USA) containing 10% fetal bovine serum with 100 μg/ml penicillin/streptomycin at 37 °C with 5% CO_2. All animal care and procedures were conducted according to the protocols and guidelines approved by the University of Ulsan Animal Care and Use Committee (UOUACUC). Standards were approved by the latter Committee (UOU-2012II-011). Bone marrow cells were isolated from 4–5-week-old C57BL/6J, hemoxygenase-1$^{-/-}$ (HO-1-KO), and HO-1$^{+/+}$ (WT) mice as described [19]. HO-1-KO mice on a Balb/c background were provided via the UOUAC and were a kind gift of Dr. MA Perrellu, Brigham and Women's Hospital and Harvard Medical School. Genomic DNA was extracted from the tails of mice for genotyping by PCR. Femora and tibiae were removed aseptically and dissected free of adherent soft tissue. The bone ends were cut, and the marrow cavity was flushed out with α-MEM from one end of the bone using a sterile 21-gauge needle and further agitated using a Pasteur pipette to generate a single cell suspension. The resulting bone marrow suspension was washed twice, and incubated on plates along with M-CSF (20 ng/ml) for 16 h. Non-adherent cells were harvested and layered on a Ficoll-Hypaque gradient, and the interface cells were collected and cultured for two more days, at which time large populations of adherent monocyte/macrophage-like cells had formed, as described before [20]. The small numbers of non-adherent cells were removed by washing the dishes with phosphate-buffered saline (PBS), and the remaining adherent cells (BMMs) were harvested and seeded in plates. A sample of the cells was analyzed with a FACSCalibur flow cytometer (Becton Dickinson, Franklin lakes, NJ) and found to be negative for CD3 and CD45R, and positive for CD11b. The absence of contaminating stromal cells was confirmed by lack of growth without addition of M-CSF. Additional medium containing M-CSF and RANKL (40 ng/ml) was added, and the medium was replaced on day 3. After incubation for the indicated times, cells were fixed in 10% formalin for 10 min, and stained for TRAP as described [24]. Numbers of TRAP-positive multinucleated cells (MNC) (three or more nuclei) were scored. BMMs were transfected with the mmu-miR-183 mimic or mmu-miR-183 inhibitors with its corresponding control (con-mimic or con-inh, respectively) (Ambion, Austin, Texas, USA) using Lipofectamine RNAiMAX transfection reagent (Invitrogen Carlsbad, CA, USA) according to the manufacturer's instructions, and further analyzed. For the efficiency and duration of miR-183 inhibition, RAW264.7 cells were transfected with 30 nM mmu-miR-183 inhibitor or its corresponding control and WT-psiCHECK2-HO-1 3′UTR using Lipofectamine 3000 (Invitrogen) according to the manufacturer's protocol. Luciferase (Renilla and firefly) assays were performed using the dual-luciferase reporter assay system (Promega, Madison, WI, USA) with the harvested cells. Relative luciferase activity was significantly lower in miR-183-inh-transfected cells than in con-inh-transfected cells from 24 h to 96 h (Supplementary Fig. 1). OCs were characterized by assessing their ability to form pits on dentine slices, as described [21]. Mature OCs were generated by incubation with M-CSF and RANKL for 5 d. Then, after treatment with EDTA, the cells were harvested [22]. Samples containing 1000 cells were seeded on dentine slices and incubated for 1 d with M-CSF and RANKL. The slices were cleaned by ultrasonication in 1 M NH_4OH to remove adherent cells and stained with Mayer's hematoxylin (Sigma) to visualize resorption pits.

2.3. Knock-down and over-expression of HO-1

BMMs were transfected with small interfering RNA (siRNA) against HO-1 (siHO-1: sc-35555, Santa Cruz Biotechnology) or scrambled siRNA (scRNA) (sc-37007, Santa Cruz Biotechnology) using Lipofectamine™ RNAiMAX (Invitrogen, No. 13778-075). Lipofectamine™ RNAiMAX (2 μl) was first diluted in α-MEM (50 μl) without serum, mixed with an equal volume of α-MEM containing 30 nM of siRNA, and incubated for 20 min. The medium was removed and fresh medium without serum was added. The resulting RNAiMAX/siRNA was added directly to the cells, giving a final volume of 700 μl. After 8 h incubation, the cells were re-fed with serum-containing medium and cultured for the indicated times.

BMMs were transfected with overexpression plasmids, pCMV6-hmox1 (MC207301, Origene) using Lipofectamine 3000 (Life Technologies) as per the manufacturer's protocol. Empty plasmid (pCMV6) was used as the negative control. Lipofectamine 3000 (1.5 μl) was diluted in Opti-MEM medium (50 μl) (Invitrogen). At the same time, 4 μg plasmid DNA was diluted in Opti-MEM (50 μl) with P3000 reagent (2 μl). The diluted Lipofectamine and plasmid DNA were then mixed, incubated for 5 min at room temperature, and distributed onto the plated cells. RNA was collected at 48 h after transfection. HO-1 mRNA was analyzed to be increased 2.1-fold in pCMV6-hmox1-transfected cells, compared with that in pCMV6-transfected cells.

2.4. Construction of the HO-1 3′UTR reporter

The HO-1 3′UTR element including the predicted miR-183 binding site was PCR-amplified. The forward primer was 5′ ctc gag aag aaa gct ttt ggg gtc cct 3′, and the reverse primer was 5′ gcg gcc gcc cac ccc ctc

Fig. 1. RANKL-induced osteoclastogenesis induces expression of miR-183. A–D. BMMs were stimulated with RANKL (40 ng/ml) and M-CSF (20 ng/ml) for the indicated times. Total RNA was extracted, and miRNA qPCR was performed to measure the expression of miR-155, miR-183, miR-122, miR-217, miR-377, miR-872 (0, 48 h) (A), and miR-183 (0, 8, 24, 48, 72 h) (D). BMMs were incubated with M-CSF and RANKL. After the indicated times, the cells were fixed and numbers of TRAP-positive MNCs per well were counted. ***$P < 0.001$ compared with 0 h cells (B). The RNA was also subjected to qPCR analysis to measure the expression of TRAP (C). The expression levels before RANKL treatment were set at 1. *$P < 0.05$; ***$P < 0.001$ compared to 0 h cells. E–H. BMMs were transfected with miR-183-mimic or miR-183-inh and the corresponding controls. The expression of miR-183 (E) and TRAP (F) and the number of TRAP-positive MNCs (G) were determined after 48 h (E, F) and 72 h (G, H). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ compared to the corresponding control. Scale bar: 200 μm in representative photos of OCs (H). Similar results were obtained in three independent experiments.