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Simvastatin inhibits osteoclast differentiation induced by bone morphogenetic protein-2 and RANKL through regulating MAPK, AKT and Src signaling

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ABSTRACT

The mevalonate pathway plays a crucial role in bone metabolism. Here we examined roles of simvastatin in osteoclast function and differentiation induced by RANKL and BMP-2 using mouse macrophage-like MLC-6 cells and human osteoclast precursor cells. MLC-6 cells expressed BMP type-I and -II receptors and Smads as well as osteoclast markers including TRAP, RANK, cathepsin-K, M-CSF receptor, MMP-9 and calcitonin receptor. Treatment with RANKL and BMP-2 acted synergistically to stimulate RANK, TRAP and cathepsin-K expression in MLC-6 cells. Simvastatin suppressed osteoclastic activity shown by increases in RANK, TRAP and cathepsin-K expression induced by RANKL and BMP-2. In contrast simvastatin alone had no effects on the osteoclastic markers in MLC-6 cells. Simvastatin activated ERK, SAPK/JNK and AKT pathways and inactivated Ras in MLC-6 cells. Simvastatin had no effect on BMP-induced Smad1/5/8 phosphorylation regardless of RANKL stimulation. Since chemical inhibition of ERK, SAPK/JNK and AKT increased TRAP and cathepsin-K expression induced by BMP-2 and RANKL, these pathways are functionally involved in inhibition of osteoclastic activity. In addition, Src phosphorylation induced by RANKL, which is involved in osteoclast differentiation, was suppressed by simvastatin. We further confirmed an inhibitory mechanism of simvastatin on osteoclast differentiation using human osteoclast precursor cells which express BMP receptor and Smad signaling machinery. Simvastatin also activated ERK pathways and inactivated Src phosphorylation in human osteoclasts differentiated by M-CSF and RANKL treatments. The inhibition of TRAP and RANK expression by simvastatin was reversed by ERK inhibition, whereas Src inhibitor enhanced simvastatin-induced suppression of osteoclast markers. Collectively, our data show that simvastatin inhibits osteoclastic differentiation through inhibiting Src as well as enhancing MAPK/AKT pathways.

1. Introduction

Osteoclasts primarily involved in bone resorption are multinucleated cells derived from hematopoietic precursors of monocyte/macrophage series [1]. Osteoclasts are bone cells that reabsorb bone tissue by removing the mineralized matrix. These cells are characterized by high expression of tartrate-resistant acid phosphatase (TRAP) and cathepsin-K [2]. Numerous inflammatory cytokines have been linked to the formation of osteoclasts including receptor activator of nuclear factor κB ligand (RANKL), tumor necrosis factor (TNF)-α, interleukin-1 (IL-1), IL-6 and macrophage-colony stimulating factor (M-CSF) [3–5]. RANKL is a membrane-bound protein of the tumor necrosis factor (TNF) ligand superfamily that is expressed on the osteoblast cell surface and has been shown to play a major role in an osteoclast differentiation cooperative with M-CSF [6]. RANKL binds to its receptor, called receptor activator of nuclear factor κB (RANK) and initiates a cascade of signaling events including c-Jun N-terminal kinase (JNK) and nuclear factor-κB (NF-κB), ultimately leading to osteoclast differentiation. RANKL activates mature osteoclasts *in vitro* and leads rapidly to resorption of bone *in vivo* by activating preexisting osteoclasts [7,8].

Besides these molecules, transforming growth factor (TGF)-β superfamily members have been demonstrated to regulate osteoclast differentiation although the action remains controversial as both inhibitory and stimulatory effects on osteoclastogenesis and osteoclast function have been reported. [9–12]. Cells belonging to the osteoclast lineage express serine/threonine kinase receptors mediating the signal of members of the TGF-β superfamily; however, little is known about the actions of growth factors in the TGF-β superfamily

Abbreviations: ActRI and ActRII, activin type-I and type-II receptor; ALK, activin receptor-like kinase; BMP, bone morphogenetic protein; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; M-CSF, macrophage-colony stimulating factor; OPC, osteoclast precursor cell; RANKL, receptor activator of nuclear factor κB; RANKL, RANK ligand; SAPK/JNK, stress-activated protein kinase c-Jun NH2-terminal kinase; TGF-β, transforming growth factor-β; TRAP, tartrate-resistant acid phosphatase.

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other than TGF-β on osteoclastic bone resorption. Therefore, elucidating the role of bone morphogenetic protein (BMP) in osteoclast function is important for understanding the possible relationship between osteoclastic bone resorption and BMP actions observed in the BMP-induced endochondral ossification process.

BMPs have established critical roles in governing various aspects of embryological development, including brain, heart, kidney and eyes [13]. BMPs also play pivotal regulatory role in mesoderm induction and dorso-ventral patterning of developing limb buds and are known to promote differentiation of mesenchymal stem cells into chondrocytes and osteoblasts as well as the differentiation of osteoprogenitor cells into osteoblasts [14]. The biological functions of BMPs are mediated through the Smad signal transduction pathway via BMP receptors. In addition to the established developmental actions of BMPs, a variety of physiological BMP actions in many endocrine and vascular tissues including the ovary [15,16], pituitary [17], thyroid [18], adrenal [19–21], kidney [22] and vascular smooth muscle cells [23] have been discovered.

Among various compounds from natural sources screened, Mundy et al. [24] found that statins enhance new bone formation in part through increased expression of BMP-2. This effect was shown to be mediated through the action of statins on osteoblast [25]. Other investigators have shown that statins may also mediate their effect on bone through osteoclasts, thus causing suppression of bone resorption [26]. Since statins affect osteoblasts and osteoclasts, and osteoblasts regulate osteoclast activity through the secretion of RANKL, we attempted to elucidate the effect of simvastatin on RANKL-stimulated signaling and osteoclastogenesis. To this end, we utilized a lipophilic simvastatin and focused on osteoclast differentiation and/or function using mouse osteoclast-like MLC-6 cells and human osteoclast precursor cells. This study shows that simvastatin inhibits BMP-induced osteoclast differentiation by regulating the RANKL/BMP-to-MAPK/AKT/Src pathway, suggesting a potential roles of simvastatin in ameliorating bone damage related to excess bone resorption.

2. Materials and methods

2.1. Reagents and supplies

McCoy's 5A medium was purchased from Invitrogen Corp. (Carlsbad, CA). Penicillin–streptomycin solution and simvastatin were purchased from Sigma-Aldrich Co. Ltd. (St. Louis, MO). Recombinant murine soluble receptor activator of NF-κB ligand (RANKL) was obtained from PeproTech EC Ltd. (London, UK) and recombinant human BMP-2 was purchased from R&D Systems, Inc. (Minneapolis, MN). Simvastatin was converted to the active form by alkaline hydrolysis. U0126, SB203580 and LY294002 were purchased from Promega Corp. (Madison, WI), and SP600125 was from Biomol Lab. Inc. (Plymouth Meeting, PA), and SH-5, Y-27632 and PP2 were from Calbiochem (San Diego, CA).

2.2. MLC-6 and human osteoclast cell culture

The mouse osteoclast-like cell line, MLC-6 was obtained from RIKEN BRC cell bank (Tokyo, Japan). MLC-6 cells were established by Dr. Sakiyama from mouse bone marrow by co-culturing bone marrow cells with mouse chondrocytes [27]. MLC-6 cells were cultured in McCoy'5A medium supplemented with 20% fetal calf serum (FCS) and penicillin–streptomycin solution at 37 °C under a humid atmosphere of 95% air/5% CO_2. Human osteoclast precursor cells (OPCs) were purchased from Lonza Walkersville, Inc. (Walkersville, MD). OPCs were cultured in 12-well fibronectin-coated plates (Biocoat®, BD-Falcon) with osteoclast precursor growth medium (OPGM) supplemented with recombinant human M-CSF and RANKL at 37 °C under a humid atmosphere of 95% air/5% CO_2. OPGM were made by adding the FBS, L-glutamine and penicillin–streptomycin to osteoclast precursor basal medium using OCGM Bullet Kit® (Lonza Walkersville, Inc.). Changes in cell morphology were monitored using an inverted microscope.

2.3. RNA extraction and quantitative real-time RT-PCR analysis

To prepare total cellular RNA, MLC-6 was cultured in 12-well plates (5×10^5 viable cells) and treated with indicated concentrations of BMP-2, RANKL and simvastatin in serum-free medium either alone or in combination with indicated concentrations of U0126, SB203580, SP600125, SH-5, LY294002, Y-27632 and PP2. Human OPCs were cultured in 12-well fibronectin-coated plates (1×10^5 viable cells) and treated with indicated concentrations of BMP-2 and simvastatin in OPGM supplemented with M-CSF and RANKL either alone or in combination with indicated concentrations of U0126 and PP2. After 24-h culture, the medium was removed, and total cellular RNA was extracted using TRIzol® (Invitrogen Corp., Carlsbad, CA), quantified by measuring absorbance at 260 nm, and stored at −80 °C until assay. The extracted RNA (1.0 μg) was subjected to an RT reaction using the First-Strand ®cDNA synthesis system® (Invitrogen Corp.) with random hexamer (2 ng/μl), reverse transcriptase (200 U), and deoxynucleotide triphosphate (0.5 mM) at 42 °C for 50 min and 70 °C for 10 min. Subsequently, hot-start PCR was performed using MgCl2 (1.5 mM), dNTP (0.2 mM), and 2.5 U of Taq DNA polymerase (Invitrogen Corp.) under the conditions we have reported [28–31]. Aliquots of PCR products were electrophoresed on 1.5% agarose gels, visualized after ethidium bromide staining, photographed, and scanned. Oligonucleotides used for RT-PCR were custom-ordered from Invitrogen Corp. PCR primer pairs were selected from different exons of the corresponding genes to discriminate PCR products that might arise from possible chromosome DNA contaminants. The primer pairs for BMP receptors, Smads and a house-keeping gene ribosomal protein-L19 (RPL19) were selected as we reported [29–31]. For RANK, TRAP, cathepsin-K, M-CSF receptor (MCSF-R), matrix metalloproteinase (MMP)-9, calcitonin receptor (CTR), glucocorticoid receptor (GCR), estrogen receptors (ERs) and peroxisome proliferator-activated receptors (PPARs), the following primer sets were utilized: mouse RANK, 861-882 and 1108-1129 (from GenBank accession No. AF019046); human RANK, 872-893 and 1134-1155 (NM_003839); mouse TRAP, 689-709 and 861-882 (NM_007388); human TRAP, 236-256 and 436-455 (J04430); mouse cathepsin-K, 551-571 and 803-823 (NM_007802); mouse MCSF-R, 1321-1330 and 1560-1580 (NM_001037859); mouse MMP-9, 380-400 and 601-621 (NM_013599); mouse CTR, 720-740 and 939-960 (BC119272); GCR, 1895-1915 and 2171-2191 (X04435); ERα, 1523-1543 and 1737-1757 (NM_007956); ERβ, 701-721 and 1004-1024 (NM_010157); PPARα, 601-622 and 932-953 (NM_011144); and PPARγ, 350-371 and 721-742 (NM_011146). For the quantification of RANK, TRAP, cathepsin-K and RPL19 mRNA levels, real-time PCR was performed using LightCycler-FastStart DNA Master SYBR Green I system® (Roche Diagnostic Co., Tokyo, Japan) under conditions of annealing at 60 to 62 °C with 4 mM $MgCl_2$, following the manufacturer's protocol. Accumulated levels of fluorescence were analyzed by the second derivative method after the melting curve analysis (Roche Diagnostic), and then, following the assay validation by calculating each amplification efficiency, the expression levels of each target gene were standardized by RPL19 level in each sample.

2.4. Western immunobloting analysis

MLC-6 cells (2×10^5 viable cells) were precultured in 12-well plates in McCoy's5A medium containing 20% FCS for 48 h. Human OPCs (1×10^5 viable cells) were precultured in 12-well fibronectin-coated plates in OPGM containing M-CSF and RANKL for 7 days. After preculture, medium was replaced with serum-free fresh medium, and then indicated concentrations of BMP-2, RANKL and simvastatin were added to the culture medium. After stimulation with growth factors for indicated periods, the membrane fraction of MLC-6 cells was