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Mevinolin enhances osteogenic genes (ALP, type I collagen and osteocalcin), CD44, CD47 and CD51 expression during osteogenic differentiation

Hyung Keun Kim [a,b,1], Sang Gwon Cho [a,1], Ji Hyun Kim [a,b], Thi Kim Phuong Doan [a,b,c], Qin Sheng Hu [a,b,c], Rehan Ulhaq [a], Eun Kyoo Song [a], Taek Rim Yoon [a,b,c,*]

[a] Department of Orthopaedics, Chonnam Natl. Univ. Hwasun Hospital, 160 Ilsimri, Hwasun-gun, Jeonnam, 519-809, Republic of Korea
[b] Cardiovascular Research Institute of Chonnam Natl. Univ. Gwangju, 501-757, Republic of Korea
[c] Brain Korea 21 Project Biomedical Human Resources Center, Chonnam Natl. University Jeonnam, 519-809, Republic of Korea

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ABSTRACT

Aims: In this study, we evaluated the effect of mevinolin on the expressions of osteogenic genes and surface molecules expression during osteogenesis.
Main methods: D1 cells were cultured in osteogenic differentiation medium (ODM) for 6 days, treated with mevinolin for 2 days, and then subjected to alizarin red S staining, MTT assays, alkaline phosphatase (ALP) activity determinations, energy dispersive X-ray spectrophotometry (EDX), real-time PCR, Western blot, fluorescence microscopy and FACS analysis.
Key findings: Mevinolin is commonly prescribed and widely used to lower cholesterol levels, and offers an important, effective approach to the treatment of hypercholesterolemia and arteriosclerosis. However, the direct effect of mevinolin on osteogenesis *in vitro* has not been clarified. ODM has been previously shown to increase the osteoblast differentiation of D1 cells. In the present study, we investigated the expressions of osteogenic genes and surface molecules during osteoblast differentiation induced by mevinolin. We found that the induction of ALP, type I collagen, osteocalcin, CD44, CD47 and CD51 by mevinolin is responsible for the osteoblastic differentiation of D1 cells.
Significance: Our data show that mevinolin enhances the expressions of proteins and surface molecules related to osteogenesis.

Introduction

Mesenchymal stem cells (MSCs) derived from bone marrow have been used to repair skeletal bone and hard tissues. Because of their multilineage potential and plasticity, MSCs can commit to form various non-hematopoietic tissues, such as, bone, cartilage, tendons and ligaments (Owen et al., 1987; Pittenger et al., 1999; Fibbe 2002; Vinck et al., 2005). In the present study, we used D1 cells, a mesenchymal stem cell line cloned from Balb/c mouse bone marrow stromal cells (Dahir et al., 2000), which are known to differentiate to the osteogenic lineage when cultured in osteogenic differentiation medium (ODM) containing ascorbic acid, dexamethasone, and β-glycerolphosphate (Vinck et al., 2003; Minguell et al., 2001; Chen et al., 2005; Li et al., 2005).

Statins inhibit cholesterol biosynthesis by competitively inhibiting the enzyme 3-hydroxyl-3-methylgutaryl-coenzyme A (HMG-CoA) reductase, which converts 3-hydroxy-3-methylglutaryl-coenzyme A to mevalonate (Goldstein and Brown, 1990). Recently, statins were shown to reduce osteoclast activity and to stimulate osteoblast differentiation *in vitro* and bone formation *in vivo* (Mundy et al., 1999; Maeda et al., 2001; Oxlund and Andreassen, 2004). Mevinolin is a statin with cholesterol-lowering and potential antineoplastic activity that was originally isolated from *Aspergillus terreus*. Mevinolin and its relatives, fluvastatin, pravastatin, and simvastatin, competitively inhibit HMG-CoA reductase, an enzyme found in Eukarya, Archaea and some bacteria, and mevinolin is used to synthesize mevalonic acid from acetyl-CoA (Cabrera et al., 1986; Lam and Doolittle, 1989). More specifically, in humans, treatment with mevinolin or its congeners helps lower cholesterol, whereas in Archaea these agents can block the production of isoprenoid lipids (the major lipid component of the plasma membrane) and completely halt growth (Cabrera et al., 1986).

The osteoporosis and osteonecrosis associated with systemic lupus erythematosus and similar diseases induce high lipid levels or hypercholesterolemia (Julian et al., 1992; Gordon et al., 2002),

* Corresponding author. Department of Orthopaedics, Chonnam Natl. Univ. Hwasun Hospital, 160 Ilsimri, Hwasun-gun, Jeonnam, 519-809, South Korea. Tel.: +82 61 379 7677; fax: +82 61 379 7681.
E-mail address: tryoon@jnu.ac.kr (T.R. Yoon).
[1] These authors contributed equally.

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and conversely, lipid-lowering agents that reduce lipid levels in the blood can be used to either prevent osteoporosis and osteonecrosis or treat these diseases during their early stages. Furthermore, lipid metabolism alterations have been implicated in the pathophysiology of these diseases, and various lipid-lowering agents may inhibit these disease processes. An animal study by Motomura et al. showed that lipid-lowering agents in combination with anticoagulants lower the risk of corticosteroid associated osteonecrosis (Motomura et al., 2004), and Pritchett, who studied statins in 284 osteonecrosis patients taking high-dose steroids, found that statins might offer protection against osteonecrosis when steroid treatment is necessary (Pritchett, 2001). Moreover, because vasodilatory and statin-based treatments are relatively free of adverse events, these agents have also been used for prophylaxis in patients who require corticosteroids for osteoporosis, osteonecrosis, or hypercholesterolemia.

In this study, we investigated whether mevinolin regulates the differentiation and function of osteoblasts by utilizing a mouse multipotent bone marrow stromal cell line. The results obtained suggest that mevinolin enhances the expressions of osteogenic genes (ALP, type I collagen and osteocalcin) and proteins (ALP and osteocalcin) by enhancing the expression of surface molecules, such as, CD44, CD47 and CD51 during osteogenesis.

Materials and methods

Osteogenic differentiation

The D1 cells used in this study were cloned from multipotent bone marrow stromal cells, as previously described (Dahir et al., 2000). D1 cells were purchased from ATCC (Manassas, VA) and maintained in DMEM containing 10% FBS (Gibco, BRL, CA) and antibiotics (Gibco). Cells were seeded at 1×10^4 cells/cm^2 and maintained in culture for 3 days in a humidified 5% CO_2 atmosphere at 37 °C. Experiments were performed after cells had reached about 80% confluence. To induce osteogenic differentiation, culture media were changed at 3 days to ODM (DMEM supplemented with 50 μg/ml ascorbic acid, 10^{-8} M dexamethasone, and 10 mM β-glycerolphosphate (all from Sigma-Aldrich, MO)). After culture for another 3 days, one group was cultured only for ODM, while another group was cultured for ODM plus mevinolin (0.1, 1 or 10 μM). Cells were then analyzed 24 or 48 h later.

Alizarin red-S staining

Calcification deposits on cells were quantified as described by Chen et al. (Chen et al., 2005). Briefly, cell cultures were washed twice with distilled water, fixed for 1 h in ice-cold 70% ethanol, and rinsed twice with deionized water. Cultures were stained for 10 min with alizarin red S, and excess dye was removed gently using running water. Calcification deposits, which appeared bright red, were identified by light microscopy and photographed. Osteogenic differentiation was quantified by determining densities and areas of alizarin red S staining using an image analysis program (Multi Gauge V3.0, Fujifilm, Japan).

Alkaline phosphatase (ALP) activity assays

Specific ALP activities were assayed based on the release of *p*-nitrophenol from *p*-nitrophenyl phosphate using LabAssay™ ALP assay kit (Wako, Japan). Optical densities of the *p*-nitrophenol produced were read at 405 nm using an Infinite M200 microplate reader (Tecan Austria GmbH, Austria). ALP activities were normalized with respect to total protein content, which was determined using a microplate reader and Bradford assay kits (Bio-Rad, CA).

Real-time-PCR analysis

To assess the effects of mevinolin on the transcriptions of genes encoding ALP (5'-ACA CCT TGA CTG TGG TTA CTG CTG A-3' (sense); 5'-CCT TGT AGC CAG GCC CGT TA-3' (antisense)), type I collagen I (5'-ACT CAG CCG TCT GTG CCT CA-3' (sense); GGA GGC CTC GGT GGA CAT TA-3' (antisense)), osteocalcin (5′-GAG GGC AAT AAG GTA GTG AAC AGA-3′ (sense); 5′-AAG CCA TAC TGG TCT GAT AGC TCG-3′ (antisense)), and on the housekeeping enzyme glyceraldehydes-3-phosphate dehydrogenase (GAPDH, 5'-AAA TGG TGA AGG TCG GTG TG-3' (sense); 5'-TGA AGG GGT CGT TGA TGG-3' (antisense)), D1 cells grown to 70% confluence on plates with/without mevinolin were homogenized using TRIzol reagent (Life Technologies, CA). Total RNA was then isolated, and 0.5 μg RNA aliquots were reverse transcribed in 20 μl buffer containing; AMV reverse transcriptase 5X, 2.5 μM poly dT, 1 mM each of dATP, dCTP, dGTP, and dTTP, 20 U of RNAse inhibitor, and 20 U of AMV RT. Reverse transcription was performed using the following conditions; initial incubation at room temperature for 10 min and then subjected to 42 °C for 15 min, 99 °C for 5 min and at 5 °C for 5 min in a GeneAmp PCR System 2700 (Applied Biosystems, CA). Aliquots of cDNA were amplified in AccuPower® GreenStar qPCR premix (Bioneer Co., Korea) using an ExiCycler™ 96 Real-time Quantitative Thermal Block (Bioneer Co., Korea).

Western blot analysis

Cell were harvested, washed twice with ice-cold PBS, and lysed in RIPA buffer (Sigma-Aldrich, MO) supplemented with protease inhibitor cocktail (Sigma, MO) for 1 h on ice with vortexing every 10 min. Lysates were centrifuged at 8000 ×*g* for 20 min to remove insoluble material, and protein concentration in supernatants were determined using Bradford assay kits using BSA as a standard. Equal amount of protein were separated on 12% SDS-PAGE gels, and blots were subsequently transferred to nitrocellulose membranes (Hybond C, Amersham Life Science, Sweden). Membranes were treated with primary antibodies against ALP, osteocalcin and β-actin (Santa Cruz Biotechnology Inc., CA), and secondary antibodies used at 1:500–1:1000. Proteins were detected by an enhanced chemiluminescence reagent using a commercial kit (Amersham Life Science, Sweden). Protein expressions were quantified by determining blot densities using an LAS-3000 system (Fujifilm, Japan).

Energy dispersive X-ray spectrometry (EDX) analysis

Energy dispersive X-ray spectrometry (EDX) was used to quantify calcium deposits. Briefly, cells were treated with different concentrations of mevinolin for 48 h, and then culture dishes were embedded in paraffin. EDX was performed using 12 mm sections. Sections were fixed to a sample holder using a conductive carbon ribbon. Maps of calcium distributions were acquired at 20 kV, and calcium levels were quantified using the EDX analysis program supplied with the Hitachi S-4700 scanning electron microscope (Hitachi, Japan).

Immunofluorescence staining

D1 cells grown on coverslips, were fixed with 4% paraformaldehyde in PBS for 15 min, permeabilized with 0.1% Triton X-100 for 15 min, and then blocked with 5% BSA in PBS for 30 min. Coverslips were then incubated with primary antibodies against mouse CD44, CD47 or CD51 (eBioscience, CA) at a dilution of 1:100, and against mouse CD45 (eBioscience) at 1:100, both at room temperature for 1 h. CD45 (a hematopoietic cell marker) was used as a negative control for MSCs. Cells were then washed with PBS and mounted in