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Simvastatin enhances human osteoblast proliferation involved in mitochondrial energy generation

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ABSTRACT

Simvastatin has been shown to stimulate osteogenic cell differentiation. Our previous study showed osteoblasts on trabecular surface are increased by simvastatin treatment in animal study. However, whether simvastatin stimulates osteoblast proliferation and by what molecular mechanism have not been adequately investigated. Because the mitochondrial function is crucial for cell survival and proliferation, we hypothesize that simvastatin may promote human osteoblast (hOBs) proliferation and it may be related to mitochondrial function. Our results showed that simvastatin significantly enhanced proliferation and increased both mRNA and protein levels of cyclin D2, Bcl-2 and the ratio of Bcl-2 to Bax (Bcl-2/Bax). Furthermore, simvastatin increased mitochondrial activity and ATP content of hOBs. Most importantly, treatment with ATP synthase blocker, oligomycin, significantly decreased both simvastatin-stimulated ATP content and cell proliferation, and completely reversed the simvastatin-induced up-regulation of cyclin D2 and Bcl-2 expression in hOBs. On the other hand, rotenone, the complex I blocker, also partially blocked simvastatin-stimulated ATP content and cell proliferation, but the blocker did not suppress the effect of simvastatin on cyclin D2 and Bcl-2 expression. These results indicate that the up-regulation of cyclin D2 and Bcl-2/Bax by simvastatin depends on the intact function of ATP synthase in the mitochondria of hOBs. It suggests that simvastatin may promote hOB proliferation, at least partly, via up-regulating mitochondrial function and subsequently cyclin D2 and Bcl-2/Bax expression. The findings provide new information for the basic medical science in bone physiology and for new therapy strategy of simvastatin on bone formation in future.

1. Introduction

Simvastatin, a hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor, is known to inhibit cholesterol biosynthesis (Horiuchi and Maeda, 2006; Jadhav and Jain, 2006; Pasco et al., 2002; Solomon et al., 2005; Svejda, 2006; van Staa et al., 2001; von Stechow et al., 2003). Accumulating reports have indicated that simvastatin has advanced effects on bone formation in vivo and in vitro (Baek et al., 2005; Maeda et al., 2001; Mundy et al., 1999; Oxlund and Andreassen, 2004; Song et al., 2003; Sonobe et al., 2005; von Stechow et al., 2003; Yazawa et al., 2005; Yin et al., 2012). Although several studies have shown that BMP-2 up-regulation in osteoblasts by the statins contributes to statin-induced bone formation (Maeda et al., 2001; Mundy et al., 1999; Song et al., 2003), the underlying mechanisms remain unclear. In a previous study, we showed that simvastatin attenuates ovariectomy (OVX)-induced osteopenia in rats (Ho et al., 2009). Most importantly, we found that simvastatin significantly increases the number of osteoblasts on the tibial trabecular bone surface in OVX rats. Another report also indicated that simvastatin increases the proliferation of mouse calvarial cells (Hwang et al., 2004). Accordingly, it is likely that, other than promoting osteogenic differentiation, simvastatin may also promote osteoblast proliferation to enhance bone formation.

Cell cycle regulation is crucial to complete cell proliferation. The cell cycle is a multi-stage process, including the G0, G1, G2, S and M phases, and the proceeding of cell cycle is regulated by a variety of regulators, such as retinoblastoma protein (Rb), cyclins,

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cyclin-dependent kinases (CDKs) and cyclin-dependent kinases inhibitors (CDKIs). CDK-cyclin complexes regulates cell cycle progression, while CDKIs inhibit the activities of CDKs (Goldberg et al., 1996; Niculescu et al., 1998; Piazza et al., 1997; Shiff et al., 1996). Reports have indicated that simvastatin promotes cell proliferation in primary cultures of mouse calvarial cells and periodontal ligament cells (PDL) (Hwang et al., 2004; Yazawa et al., 2005). However, whether simvastatin affects the expression of cell cycle regulators in human osteoblasts (hOBs) has not been thoroughly investigated. Numerous studies have indicated that selectively disrupting mitochondrial function led to an inhibition of cell proliferation, demonstrating that cell division is altered by the cellular energy pool (Carreras et al., 2004; Galli et al., 2003; Gemin et al., 2005). In addition, the Bcl-2 family mainly regulates mitochondrial function and cell survival. Accordingly, we hypothesize that simvastatin may enhance mitochondrial function and cell cycle regulator to promote osteoblast proliferation. In this study, we examined the effects of simvastatin on proliferation and clarified whether this effect was due to altering gene expression of cell cycle regulators, survival factors and mitochondrial functions in hOBs.

2. Materials and methods

2.1. Ethics statement

Primary human osteoblasts (hOBs) were isolated from bone chips of 40- to 60-year-old patients (four men and four women) who were generally healthy except for hip dysplasia, which was being treated with hip arthroplasty at Kaohsiung Medical University Hospital. The protocol for this study was approved by the Institutional Review Board (IRB) at Kaohsiung Medical University, and written informed consent was obtained from each donor to produce the hOB samples.

2.2. Cell culture

The hOBs used in each replication were mixed from three independent patients, selected randomly from eight patient samples. Three replications were repeated in each experiment. The average doubling time of hOBs was 18.46 ± 0.6 h under the experimental condition (data not shown), and the primary hOBs showed similar basal proliferative rates between experiments (data not shown).The hOBs were cultured in Dulbecco modified Eagle medium (Gibco BRL, Gaithersburg, MD) containing 10% fetal bovine serum, 100 mg/ml penicillin, ascorbic acid and non-essential amino acid solution (bone medium). Cultures were incubated in a humidified atmosphere of 5% CO_2 at 37 °C (Cui et al., 1997a, 1997b; Diduch et al., 1993). For all experiments, cells were cultivated under these conditions within four passages, and the medium was changed every two days. The doubling time of human osteoblasts is 20–24 h under these experimental conditions.

2.3. Drug treatment

Simvastatin, rotenone and oligomycin were dissolved in DMSO as stock solutions. The simvastatin concentrations used in these experiments ranged from 1 nM–1 μM (Merck Sharp & Dohme Corp, Rahway, NJ). The mitochondrial respiratory chain blockers were the complex I inhibitor rotenone and the ATP synthase inhibitor oligomycin, which were used within 1–50 μM and 5–100 ng/ml, respectively. All reagents were diluted with culture medium immediately before the treatments began. The final concentration of DMSO in each treatment was limited to 0.1% or lower to reduce its influence on the cells. For the proliferation and cytotoxicity experiments, sub-confluent cells were starved in a medium containing 2% serum for 24 h. Afterwards, the cultures were maintained in medium with or without drugs.

2.4. Thymidine incorporation

Cells (2×10^3/well) cultured in 96-well plates were treated with reagents for 24 h. Four hours prior to harvesting, [^{3}H] thymidine (2 μCi/well) (Tornkvist et al., 1985) was added to the cells. The incubations were terminated by washing the cells with phosphate buffered solution (PBS). Then the cells were detached using 1% trypsin/EDTA and collected onto a 96-well UniFilter (Packard, Meriden, CT) using a FilterMate Harvester (Packard, Meriden, CT). The UniFilter was rinsed using 95% ethanol and dried for 30 min. After sealing with a TopSeal-A (Packard, Meriden, CT), liquid scintillant was added to the sealed and dried UniFilter and [^{3}H] thymidine counts were detected by TopCount microplate scintillation and luminescence counters (Packard, Meriden, CT).

2.5. Cell counting

The hOBs were seeded at a density of 3×10^4 cells/well in six-well plates and were cultivated in 2% FBS-containing medium with or without simvastatin (10 to 500 nM). After simvastatin treatments, the number of cells was determined every day for six days using a hemocytometer. Each sample was repeated for triplicate and each experiment was tested by three samples.

2.6. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Human OBs (2×10^3/well) were seeded in 96-well plates and cultured to 70–80% confluence. The metabolic activity of the hOBs was measured using the tetrazolium salt assay. A mitochondrial dehydrogenase cleaves the tetrazolium salt yielding a blue formazan, thus reflecting mitochondrial activity. Therefore, 10 μl of MTT solution (5 mg/ml in PBS as stock solution) was added to each well (MTT at a final concentration of 0.5 mg/ml) and then incubated for 4 h at 37 °C. After incubation, the supernatant was removed and 100 μl of DMSO was added to each well for 10 min to dissolve the formazan crystals. The 595-nm absorbance of each well of the plate was read using an ELISA plate reader (Bio-Rad) (Vybrant® MTT Cell Proliferation Assay Kit-1000 Assays, Life techenlogies, USA).

2.7. Cytotoxicity assayed by lactate dehydrogenase (LDH) leakage

Lactate dehydrogenase (LDH) leakage from cells was measured to quantify cytotoxicity using a cytotoxicity detection kit (Roche, Mannheim, Germany) (Crowston et al., 1998; Mesner and Kaufmann, 1997). The hOBs were seeded into 24-well plates (5000 cells/well). After drug treatment, the supernatants and cell layers of the cultures were collected for assay. The cell layers were lysed with 1% TritonX-100, and the cell lysates and supernatants were assayed separately in 96-well plates. Briefly, 100 μl of catalyst solution was added to each well for 20 min. The 490-nm absorbance of each well of the plate was measured with an ELISA plate reader (Bio-Rad Laboratories Inc., Hercules, CA). LDH leakage from hOBs was calculated using the following formula:

$$\text{LDH leakage} = A_{490}(\text{supernatant})/A_{490}(\text{supernatant} + \text{cells})$$

2.8. Real-time PCR

Total RNA from hOBs was extracted by using TRIzol® Reagent (cat. 15596026, Invitrogen, USA). First-strand cDNA was synthesized from