Simvastatin Induces Osteogenic Differentiation and Suppresses Adipogenic Differentiation in Primarily Cultured Human Adipose-Derived Stem Cells

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Abstract – Recent *in vitro* and *in vivo* animal studies have reported that statin, a cholesterol-lowering drug, stimulate osteogenic differentiation. In the present study, we investigated the effect of simvastatin on osteogenic and adipogenic differentiation in primarily cultured human adipose-derived stem cells (hADSCs). The simvastatin treatment significantly increased the positive cell numbers in alkaline phosphatase and von Kossa staining, and enhanced the expression levels of bone morphogenic protein (BMP)-2, core binding factor alpha 1 (cbfa1), collagen type I and osteonectin mRNAs. Lastly, hADSCs were cultured in the adipogenic media with or without simvastatin to examine the effect of simvastatin on adipogenic differentiation. In the RT-PCR analysis, there were notable decreases in mRNA expression of aP1, C/EBP- α and PPAR- γ in hADSCs cultivated in simvastatin-added medium, compared to those in simvastatin-free medium. It suggests that the adipogenic differentiation was significantly inhibited by simvastatin treatment. These observations indicate that simvastatin induces osteogenic differentiation and suppresses adipogenic differentiation in hADSCs.

Keywords: Simvastatin, Human adipose-derived stem cells, Osteogenic differentiation, Adipogenic differentiation

INTRODUCTION

Although bone marrow (BM) is a primary source of multipotent mesenchymal stem cells (MSCs), BM harvest is a highly invasive procedure and the number, differentiation potential, and maximal life span of MSCs from BM decline with increasing age. Since the first description by Friedenstein (Friedenstein *et al.*, 1974), much work has shown their properties *in vitro* and *in vivo* (Pittenger *et al.*, 1999; Deans and Moseley, 2000; Fibbe and Noort, 2003). MSCs have considerable therapeutic potential in pre-clinical (Kopen *et al.*, 1999; Petite *et al.*, 2000) and clinical (Horwitz *et al.*, 1999; Koç *et al.*, 2000; Chen *et al.*, 2004; Le Blanc *et al.*, 2004; Jaquet *et al.*, 2005) studies for the treatment/ regeneration of neuronal degeneration, osteo-

genesis imperfecta, graft versus host disease, support of hematopoietic engraftment, metabolic diseases, bone and cartilage tissues, as well as renal and myocardial infarction. Adipose tissue (AT), like bone marrow, is derived from the embryonic mesenchyme and contains a stroma that is easily isolated. A putative stem cell population within the adipose stromal compartment can be isolated from human lipoaspirates and, like MSCs, differentiate toward the osteogenic, adipogenic, myogenic, and chondrogenic lineages. AT can be obtained by a less invasive method and in larger quantities than BM, but few studies have tested human adipose-derived stem cells (hADSC).

Statins, inhibitors of 3-hydroxy-3-methylglutarylcoenzyme A (HMG-CoA) reductase, have revolutionized the treatment of hypercholesterolemia by effectively lowering plasma cholesterol and being well tolerated to reduce cardiovascular morbidity and mortality (Shepherd *et al.*, 1995; Vaughan *et al.*, 2000; Sugiyama *et al.*, 2000; Stancu

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and Sima, 2001; Togel *et al.*, 2005; Blinc and Poredos, 2007; Karpisek *et al.*, 2007; Lin *et al.*, 2008). Statins have activity beyond lipid-lowering effects (Vaughan *et al.*, 2000), including the potential to induce osteogenic changes on bones (Lips, 2002; Ho *et al.*, 2009). Statins promote osteoblasts to synthesize bone morphogenic protein 2 (BMP2) (Gori *et al.*, 1999; Mundy *et al.*, 1999), a growth factor that causes osteoblasts to differentiate, proliferate, mature, and form new bone in vitro and in vivo, and also inhibits adipocyte differentiation (Gimble *et al.*, 1995). Here, we investigated the effect of simvastatin on osteoblastic and adipocytic differentiation in hADSCs in vitro.

MATERIALS AND METHODS

Procurement of specimen

Adipose tissue samples were isolated from lipoaspirates obtained during elective liposuction surgery. These patients did not have significant medical or medication history. The approval for this study was obtained from the institutional review boards (IRB) of the Catholic University of Korea, College of Medicine, and informed consent was given according to the Declaration of Helsinki.

Isolation and Culture of preadipocytes

hADSC isolation was performed as described by Im (Im *et al.*, 2005). Briefly, lipoaspirates were washed three times with phosphate-buffered saline (PBS, Gibco-BRL, Grand Island, NY, USA) containing 10% antibiotic/antimycotic (P/S, Gibco-BRL) and 1% bovine serum albumin (BSA, Bovogen, Ogilvie St. Essendon Vic, Australia), and the extracellular matrix was digested with 0.06% collagenase (collagenase type I, Invitrogen Corporation, Carlsbad, CA, USA), shaken for 2 h at 37°C, followed by filtration through 40- μ m nylon mesh (Cell strainer, BD Falcon, Two Oak Park, Bedford, MA, USA). Erythrocytes were removed using erythrocyte lysis buffer (0.15 M ammonium chloride, 1.0 mM potassium bicarbonate, and 0.1 mM EDTA) and the remaining cells were seeded in culture flasks under conditions identical to preadipocytes. The cells were cultured in Minimum Essential Medium α (MEM α , Gibco-BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA) and 1% Penicillin-Streptomycin (P/S, Gibco-BRL) in a humidified atmosphere containing 5% CO₂ at 37°C. After 24 h, the cultured cells were washed with PBS to remove any nonadherent material. The medium was replaced twice a week during the expansion period. When the cells reached 80% confluence, they were detached from the

culture dish using 1X Trypsin-EDTA (Gibco BRL, Grand Island, NY, USA), and washed with PBS, followed by re-counting and re-plating. The cells were suspended in cryo-preservation medium containing 50% MEM α , 40% FBS, and 10% dimethylsulfoxide (DMSO, St. Louis MO, USA) after culturing through passage 2.

Colony-forming unit-fibroblast (CFU-F) assay

The colony-forming unit-fibroblast assay (CFU-F) was performed as described by Castro-Malaspina (Castro-Malaspina *et al.*, 1980). Briefly, hADSCs were plated onto 10 cm dishes at 4×10^6 cells (in triplicate). After 8 to 12 days, CFU-F formation was examined and the cells, stained with crystal violet, were counted.

Flow cytometry

Flow cytometry on the cell surface markers STRO-1, CD105, and CD34 was conducted to separate MSCs from hADSCs. During passage 3, the hADSCs were harvested into a test tube (Becton Dickinson, Franklin Lakes, NJ, USA) at 1×10^7 cells/ml, and washed 3 times with wash buffer (0.2% BSA, 0.1% NaN₃, 0.5 mM EDTA). The conjugated to fluorescein isothiocyanate (FITC) or phycoerythrin (PE) antibodies to CD34 (BD Bioscience) and CD105 (Serotec Ltd, Station Approach, Kidlington, Oxford, UK), were treated for an hour and washed 3 times with wash buffer, followed by flow cytometry. The same procedure was used for STRO-1 (Human anti-mouse monoclonal antibody, IgM subclass; R&D system, Minneapolis, MA, USA), except with a secondary FITC antibody.

Osteogenic differentiation

During passage 3, hADSCs were plated onto 12 well plates (6×10^5 cells/well) and treated with: normal medium (complete MEM α), osteogenic differentiation medium (MEM α including 0.1 μ M dexamethasone, 10 μ M glycerol phosphate, and 50 μ M L-ascorbic acid 2-phosphate), MEM α included 100 ng/ml of rhBMP2, or 1 μ M of simvastatin. Media was replaced every three days.

Adipogenic differentiation

hADSCs at passage 3 were plated onto 6 well plates (1.5×10^6 cells/well) and cultured in adipogenic media (0.5 mM isobutyl methylxanthine, 1 μ M dexamethasone, 10 μ M insulin, 200 μ M indomethacin) with or without 1 μ M of simvastatin. Media was changed every three days.

Alkaline phosphatase (ALP) staining

Plates were washed 3 times with sterile triple-distilled water at 7, 14, 21 and 28 days after the first treatment date.

The plates were fixed with Citrate-Acetone-Formaldehyde fixer solution (Sigma; St. Louis, MO, USA), and washed 3 times with sterile triple-distilled water. They were stained with Alkaline-dye mix (Nitrite, FRV-alkaline, Naphthol AS-BI alkaline solution; Sigma) in the dark for 20 minutes at normal temperature. Cells were washed 3 times, counterstained with Hematoxylin (Sigma), and washed again 3 times with water. Plates were observed an inverted microscope (IX2-SLP; Olympus, Japan).

von Kossa and oil red O staining

Plates were washed 3 times with sterile triple-distilled water, fixed with 4% paraformaldehyde (Merck; Frankfurt-er Str., Darmstadt, Germany) for 15 minutes at normal temperature, and washed again 3 times with water. Using 1% silver nitrate (Merck; Frankfurter Str., Darmstadt, Germany) solution, the cells were stained for 30 min in the dark or for 30 min with 5% oil red O (Sigma), followed by washing. For von Kossa staining, the plates were left for 1 h under UV and counterstained with 0.1% Eosin (Sigma; St. Louis, MO, USA).

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Plates were washed with PBS (Gibco BRL) after 7, 14, 21 and 28 days of osteogenic differentiation or 7 and 14 days of adipogenic differentiation. Cells were collected using a cell scraper (Sarsted, Inc, Newton, NC, USA) and the RNA was separated with a QIAGEN RNA extraction kit (QIAGEN, Hilden, Germany), followed by RT-PCR for osteogenic or adipogenic marker primers and RT-PCR premix (Bioneer, Korea). Primers and reaction conditions

are described Table I.

Lipolysis assay

hADSCs in passage 3 were plated on 96 well plates, 5×10^4 cells/well, and treated with normal media (MEM α) or adipogenic media with or without 1 μ M simvastatin. After 7 or 14 days, the lipolysis assay was performed (Brasaemle *et al.*, 2004).

Quantitative analysis

Quantitative analysis of ALP, von Kossa, and Oil red O staining were measured by Optimas program (Meyer Instruments, Inc; Langham Creek, Houston, TX, USA) and expressed as mean percentage area $\pm$ SD.

RESULTS

Cell and colony morphology

After 12 days of culture, hADSCs exhibited a fusiform, spindle-shaped appearance (Fig. 1). The cells proliferated and formed morphologically homogenous colonies. Morphological observation at 8 h showed segregated, spindle-shaped cells starting to adhere to the bottom of the plate. Colony formation on day 12 was 5 to 8 colonies per 100 mm plate (Fig. 1A and B).

Cell sorting

FACS analysis of the surface antigens, CD 34, STRO-1, CD105, and CD166 showed no change in CD34 levels, but STRO-1 (Fig. 1C), CD105 (Fig. 1D), and CD166 (Fig. 1E) showed a positive expression of 93%, 92%, and 95%, respectively.

Table I. Used primers

	Name		Primer	Annealing
Osteogenic	Runx2/Cbfa1	Forward	5'-CCG CAC GAC AAC CGC ACC AT-3'	51°C
		Reverse	5'-CGC TCC GGC CCA CAA ATC TC-3'	
	ALP	Forward	5'-TGG AGC TTC AGA AGC TCA ACA CCA-3'	58°C
		Reverse	5'-ATC TCG TTG TCT GAG TAC CAG TC-3'	
Adipogenic	Collagen type I	Forward	5'-GGA CAC AAT GGA TTG CAA GG-3'	58°C
		Reverse	5'-TAA CCA CTG CTC CAC TCT GG-3'	
	aP2	Forward	5'-GCT TTG CCA CCA GGA AAG TG-3'	60°C
		Reverse	5'-ATG ACG CAT TCC ACC ACC AG-3'	
	C/EBP α	Forward	5'-AGA AAG GGG TGG AAA CAT AGG-3'	58°C
		Reverse	5'-GAA AGC TGA GGG CAA AGG-3'	
Control	PPAR- γ	Forward	5'-AAA CTC TGG GAG ATT CTC CT-3'	56°C
		Reverse	5'-TCT TGT GAA TGG AAT GTC TT-3'	
	GAPDH	Forward	5'-CCG CAT CTT CTT TTG CGT CGC-3'	52°C
		Reverse	5'-GCA ACT GTG AGG AGG GGA GAT TCA G-3'	

The reaction conditions were as follows: 1. incubation at 94°C for 2 min, 2. denaturation at 94°C for 45s, 3. annealing at 51°C (Runx2/Cbfa1) or 58°C (ALP and Coll) for 45s, 4. polymerization at 72°C for 60s for 32 cycles, 5. extension at 72°C for 5 min, 6. The amplified products were analyzed twice by means of 2% agarose gel electrophoresis.

Fig. 1. Conformation of mesenchymal stem cell on hADSCs by colony formation unit (CFU) assay and fluorescence-activated cell sorting (FACS). hADSCs from human adipose tissue were seeded in 100 mm culture dish. After 8 days, the cells were stained with crystal violet (A, B). During P 3, the cells were harvested. The antibodies of CD34, STRO-1, CD105 and CD166, to which fluorescein isothiocyanate (FITC) was adhered were analyzed by FACS. CD34 was used as a control (C-E). (A) hADSCs (100 mm dish), (B) hADSCs ($\times 100$), (C) stained STRO-1, (D) stained CD105 and (E) stained CD 166.

Confirmation of osteogenesis induction

Alkaline phosphatase staining (ALP): We performed ALP staining after hADSC treatment with BMP2, osteogenic differentiation media, or simvastatin for 7, 14, 21, or 28 days (Fig. 2A-D and I). ALP measures the presence of mineralization by forming a blue product. Osteogenic media (Fig. 2B), BMP2 (Fig. 2C), and simvastatin (Fig. 2D) all increased ALP staining after 7 days, suggesting the induction of osteogenic differentiation. Osteogenic differentiation media and BMP2 showed darker ALP staining with longer treatment, but simvastatin did not, confirming that no further osteogenic differentiation occurred after 7 days (Fig. 2I).

von Kossa staining: We used von Kossa staining to test whether calcium accumulation occurred during osteogenic differentiation (Fig. 2E-H and J). As for ALP staining, simvastatin treatment induced maximal von Kossa staining by day 7. Osteogenic differentiation media and BMP2 induced dramatic staining after 14 days, but the color continued to develop (Fig. 2E-H and J). Shorter simvastatin treatments (3, 6, 9, or 12 days) showed that staining appeared by day 3 and plateaued at day 9 (Fig. 3).

Osteogenic marker gene expression: Simvastatin treatment time-dependently increased mRNA expression of *cbfa1*, osteocalcin, collagen type I, osteonectin, and BMP2 (Fig. 4).

Confirmation of adipogenesis inhibition

Oil red O staining: Adipogenic media induced the differentiation of hADSCs into adipocytes, but 1 μ M simvastatin blocked this activity by about 50% (Fig. 5A-G). The adipocytes existed in clusters with cytoplasmic lipid droplets that were positive for Oil Red O stain, with fewer droplets seen after simvastatin treatment.

Adipogenic marker gene expression: Adipogenic media also time-dependently increased mRNA levels of *aP1*, *C/EBP α* , and *PPAR γ* , but 1 μ M simvastatin blocked this activity (Fig. 5H and I).

Lipolysis assay: Treatment with adipogenic media for 14 days induced higher levels of glycerol in hADSC, and 1 μ M simvastatin further increased these levels over an additional 3 days (Fig. 6).

DISCUSSION

Multipotent mesenchymal stromal cells (MSC), also called mesenchymal stem cells, are characterized by adherence to plastic when maintained in standard cultures in vitro, by expression of the surface antigens, CD105 and CD90, but a lack of the hematopoietic markers CD34 and CD45, and by differentiation into osteoblasts, adipocytes, and chondroblasts in vitro (Dominici *et al.*, 2006). A putative stem cell population within the adipose stromal com-

Fig. 2. Effects of osteogenic media, BMP-2, and simvastatin on alkaline phosphatase expression and mineralization in hADSCs ($\times 100$). During P3, the cells were separated into 12-well plate and they were incubated in MEM, OM media, 100 ng/ml of BMP-2 and 1 μ M of simvastatin, separately. After 7, 14, 21 and 28 days, the cells were stained with Alkaline-dye mix or 1% silver nitrate solution and observed with inverted microscope. MEM was used as a negative control while OM media and BMP-2 were used as a positive control. (A) Treatment of MEM after 7 days (ALP), (B) Treatment of OM media after 7 days (ALP), (C) Treatment of 1 ng/ml BMP-2 after 7 days (ALP), (D) Treatment of 1 μ M simvastatin after 7 days (ALP), (E) Treatment of MEM after 14 days (von Kossa), (F) Treatment of OM media after 14 days (von Kossa), (G) Treatment of 1 ng/ml BMP-2 after 14 days (von Kossa), (H) Treatment of 1 μ M simvastatin after 14 days (von Kossa) to (I), Quantitative analysis of alkaline phosphatase expression and (J) Quantitative analysis mineralization effect.

partment can be isolated from human lipoaspirates and, like MSCs, differentiate toward the osteogenic, adipogenic, myogenic, and chondrogenic lineages. AT can be obtained by a less invasive method and in larger quantities than BM. We separated hADSCs from lipoaspirates using flow cytometry of the cell surface markers, STRO-1, CD105, and CD34 (Fig. 1C-E). Mundy *et al.* (1999) reported the stimulation of bone formation in vitro and in rodents by statins (Mundy *et al.*, 1999). The statins are the most effective and best-tolerated agents for treating dyslipidemia. These drugs are competitive inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which catalyzes an early, rate-limiting step in cholesterol biosynthesis. Statins exert their major effect-reduction of LDL levels-through a mevalonic acid-like moiety that competitively inhibits HMG-CoA reductase.

Lovastatin inhibits adipogenic and stimulates osteogenic differentiation in BM mesenchymal cell cultures (Li *et al.*,

2003) and in mouse BM stromal cells (Song *et al.*, 2003). The inhibition of mevalonate synthesis prevents the synthesis of isoprenoid precursors (geranyl and farnesyl pyrophosphates), which are substrates for prenylation of small GTP binding proteins (Rho, Rac, Rab). This decrease in prenylation inhibits osteoclast activity because these small proteins cannot anchor to the membrane of osteoclasts because they lack a lipid chain. Statins promote osteoblasts to synthesize BMP2, a growth factor that causes osteoblasts to differentiate, proliferate, mature, and form new bone in vitro and in vivo, as well as inhibits adipocyte differentiation. Simvastatin increases bone volume and induce the expression of osteogenic markers, like BMP2, osteocalcin, osteopontin, and alkaline phosphatase (Ohnaka *et al.*, 2001; Gonyeau, 2005).

Statin users showed a trend toward fewer hip fractures and improved hip bone BMD (Bauer *et al.*, 2004), but meta-analysis of the two randomized clinical trials did not

Fig. 3. Earlier effects of simvastatin (1.0 μ M) on alkaline phosphatase expression and mineralization in hADSCs using ALP and von Kossa staining ($\times 100$). During P3, hADSCs were seeded 12-well plate and were incubated in 1 μ M of simvastatin. After 3, 6, 9 and 12 days, the cells were stained with Alkaline-dye mix or 1% silver nitrate solution, and they were observed with inverted microscope. (A-D) ALP staining, (E-H) von kossa staining and (I) Quantitative analysis of alkaline phosphatase expression and mineralization effect.

Fig. 4. Simvastatin increases osteogenic marker genes expression, such as cbfa1, osteocalcin, collagen type I, osteonectin and BMP-2. The hADSCs were treated with 1 μ M of simvastatin. After 1, 3, 6, 9 and 12 days, osteogenic marker genes expression were observed using RT-PCR.

support a protective effect on hip or non-spine fractures (Pedersen and Kjekshus, 2000; Reid *et al.*, 2001). Among the 13,592 participants in the National Health and Nutritio-

nal Examination Survey (NHANES) III who did not report the use of lipid-lowering drugs, there was no correlation between lipid levels and bone mineral density (BMD) (Solomon *et al.*, 2005). Thus, it seems unlikely that hyperlipidemia could explain the discrepancies in the bone effects of statins between observational studies and randomized controlled trials. Finally, the physical activity of patients and controls was not quantified in the studies, although it is associated with a higher BMD. Statins require higher doses animal studies (10-fold) for bone effects than are used in dyslipidemia, and long-term treatment may be required to see activity.

We found that hADSCs treated with osteogenic media and BMP2 were partially positive for ALP staining after 7 days (Fig. 2A-D), but staining was stronger in the simvastatin group and plateaued at 7 days (Fig. 2D and I), suggesting earlier osteogenic differentiation. BMP2 produced a darker red color than osteogenic differentiation media (Fig. 2A-C). von Kossa staining, a measure of calcium accumulation, showed similar results: simvastatin produced staining that plateaued after 7 days (Fig. 2H), whereas differentiation media and BMP2 color darkened over time (Fig. 2E-G). Simvastatin produced staining as early as 3 days and plateaued between 7-9 days (Fig. 3).

Simvastatin time-dependently increased mRNA levels

Fig. 5. Simvastatin inhibits adipogenic differentiation in hADSCs. The hADSCs were treated adipogenic media with or without 1 μ M of simvastatin. After 1, 7 and 14 days, hADSCs were stained with 5% Oil red O reagent and they were observed with inverted microscope. (A) After 1 day of treatment in adipogenic media, (B) After 7 days of treatment in adipogenic media, (C) After 14 days of treatment in adipogenic media, (D) After 1 day of treatment in adipogenic media with 1 μ M of simvastatin, (E) After 7 days of treatment in Adipogenic media with 1 μ M of simvastatin, (F) After 14 days of treatment in Adipogenic media with 1 μ M of simvastatin, (G) Quantitative analysis of A-F and Adipogenic marker genes expression without (H) and with (I) 1 μ M simvastatin.

Fig. 6. Lipolysis assay containing Adipocyte-derived mesenchymal cells. hADSCs were incubated in adipogenic media. After 14 days, the cells were treated with 1 μ M of simvastatin. 1, 2 and 3 days later, glycerol concentration was analyzed by ELISA method.

of cbfa1, osteocalcin, collagen type I, osteonectin, and BMP2 (Fig. 4), as well as increased ALP activity (2-fold

above basal) (Fig. 2I). Taken together, simvastatin treatment induces osteoblastogenesis.

We next measured adipocyte differentiation using Oil Red O staining and changes in aP1, C/EBP α , and PPAR γ mRNA levels (Fig. 5). Simvastatin (1 μ M) inhibited the adipocyte differentiation induced by adipogenic media by about 50%. The adipocytes existed in clusters with cytoplasmic lipid droplets that were positive for Oil Red O stain, with fewer droplets seen after simvastatin treatment (Fig. 5A-G). Simvastatin also induced lipolysis, as shown by increased glycerol levels after 3 days of treatment in differentiated hADSCs (Fig. 6).

In conclusion, our data suggest that human AT contains multipotent cells and may represent an alternative stem cell source to bone marrow-derived MSCs. Simvastatin increased osteogenic differentiation and decreased adipogenesis in hADSCs. Further studies about the effects of long term use of simvastatin on body composition and bone remodeling in humans are needed. In addition, other statins may have better affects, as some statins are lipophilic (simvastatin and lovastatin, which are prodrugs) and others are more hydrophilic (pravastatin, atorvastatin, fluvastatin). These differences may affect bone bioavailability and influence their therapeutic effects.

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