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The effect of simvastatin on the differentiation of marrow stromal cells from aging rats

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Simvastatin is a pro-drug of the potent 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor. Simvastatin inhibits cholesterol synthesis in humans and animals. Antecedent studies reported that statins could increase bone formation *in vitro* in cell lineages and primary cultured marrow stromal cells (MSCs) from juvenile mice. MSCs maintain the ability to differentiate into multiple lineages in adult life, but a decline in the stemness potential with aging has been recognized, which results in reduced osteogenesis and increased adipogenesis in the bone marrow. Thus, we assessed the effect of simvastatin on osteoblastic and adipocytic differentiation of MSCs from aging rats, 18 months of age. Simvastatin, added into culture medium with a low dose of dexamethasone, enhanced alkaline phosphatase (ALP) activity and staining, increased the gene expression of ALP and osteocalcin (OC), and promoted mineralization in a dose-dependent fashion. Simultaneously, simvastatin also decreased Oil Red O staining and inhibited the gene expression of lipoprotein lipase (LPL) and peroxisome proliferator activated receptor (PPARγ_2) in a dose-dependent fashion. Significant effects were observed at 10^{-6} M and 10^{-7} M simvastatin ($p < 0.05$). These results indicate that simvastatin has anabolic effects on bone through the inhibition of adipocytic differentiation and the modest promotion of osteoblastic differentiation, suggesting that it could be used for the treatment of common metabolic bone diseases, such as aged osteoporosis.

1. Introduction

Aging can affect the bone marrow microenvironment in multiple ways and is always accompanied by a decrease in osteogenesis and an accumulation of adipose tissue inside the marrow cavity (Rozman et al. 1989), which is related to an overall reduction of osteogenic stem cells (D'Ippolito et al. 1999). Moreover, it has also been suggested that a decline in the osteogenic capacities of marrow stromal cells (MSCs) exists in the aged, but not in the young (Bergman et al. 1996; Katzburg et al. 1999; Nishida et al. 1999). Osteogenic and adipogenic cells arise from MSCs. MSCs, as pluripotent stem cells, can differentiate under appropriate stimuli into multiple lineages, including osteoblasts, adipocytes, chondrocytes, and myocytes (Aubin 2001; Benayahu 2000). The lineage determination between osteoblasts and adipocytes may be a critical component in the reciprocal regulation of osteoblastogenesis and adipogenesis. It is possible, therefore, that the inhibition of marrow adipogenesis with a concomitant increase in osteoblastogenesis could prevent adipocyte formation or divert existing adipocytes to become more osteoblastic, with a resulting increase in functional bone cell (Nuttall and Gimble 2000).

Simvastatin is a competitive inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, a liver microsomal enzyme, and widely used for the treatment of hypercholestemia (Hamelin and Turgeon 1998). Mundy et al. first reported that simvastatin and lovastatin stimulated *in vivo* bone formation in rodents and increased new bone volume in cultures from mouse calvaria (Mundy et al. 1999). Several studies have also shown the effects of simvastatin on human osteosarcoma cells, bone marrow cells, and non-transformed osteoblastic cells (MC3T3-E1) (Maeda et al. 2003, 2001, 2004). All these studies used pluripotent cell lines or MSCs from young individuals as experimental models. However, the effect of simvastatin on bone marrow-derived mesenchymal stem cells from the aging has not been clarified. Thus, we chose MSCs from aging rats as our experimental model and examined the effect of various doses of simvastatin on osteoblastic and adipocytic differentiation of aging MSCs *in vitro*, which would help us understand whether there is a real stimulatory effect of simvastatin on bone formation in the aged with osteoporosis.

2. Investigations and results

2.1. Effect of simvastatin on osteogenic differentiation of MSCs

Confluent MSCs were further cultured in osteogenic medium consisting of β-glycerophosphate, Dex, and ascorbic acid (Jaiswal et al. 1997) with simvastatin (10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9} M) and ethanol vehicle. ALP is considered

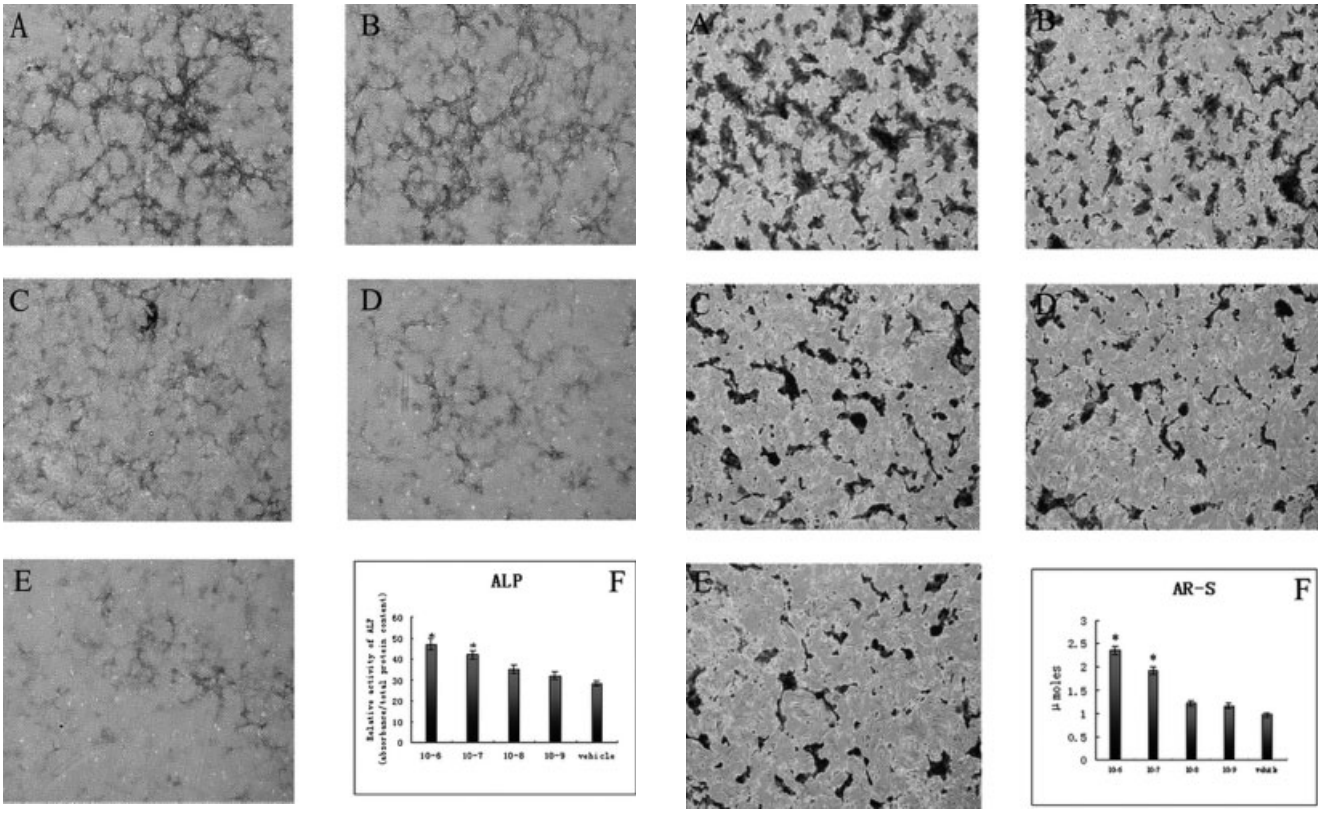

Fig. 1: ALP staining and activity of differentiating MSCs from aging rats by simvastatin.
MSCs were treated in osteoinductive medium with vehicle or graded doses of simvastatin (10^{-6} to 10^{-9} M; A–D) for 10 days. ALP-positive area are shown in dark; at the same time, ALP activity was measured with the pNPP assay and normalized on the basis of protein content per disk (F). Each point represents the mean ± SEM of 4 determinations. * $P < 0.05$ (compared with vehicle control)

Fig. 2: Effect of simvastatin on the mineralization of extracellular matrix by MSCs.
Cells were treated with vehicle or simvastatin (10^{-6} to 10^{-9} M; A–D) for 21 days. AR-S staining was performed for the demonstration of mineralized nodule formation at days 21. AR-S was then eluted from the matrix and measured by spectrophotometry at 562 nm (F). The data are expressed as the mean ± SEM of 4 determinations. * $P < 0.05$ (compared with vehicle control)

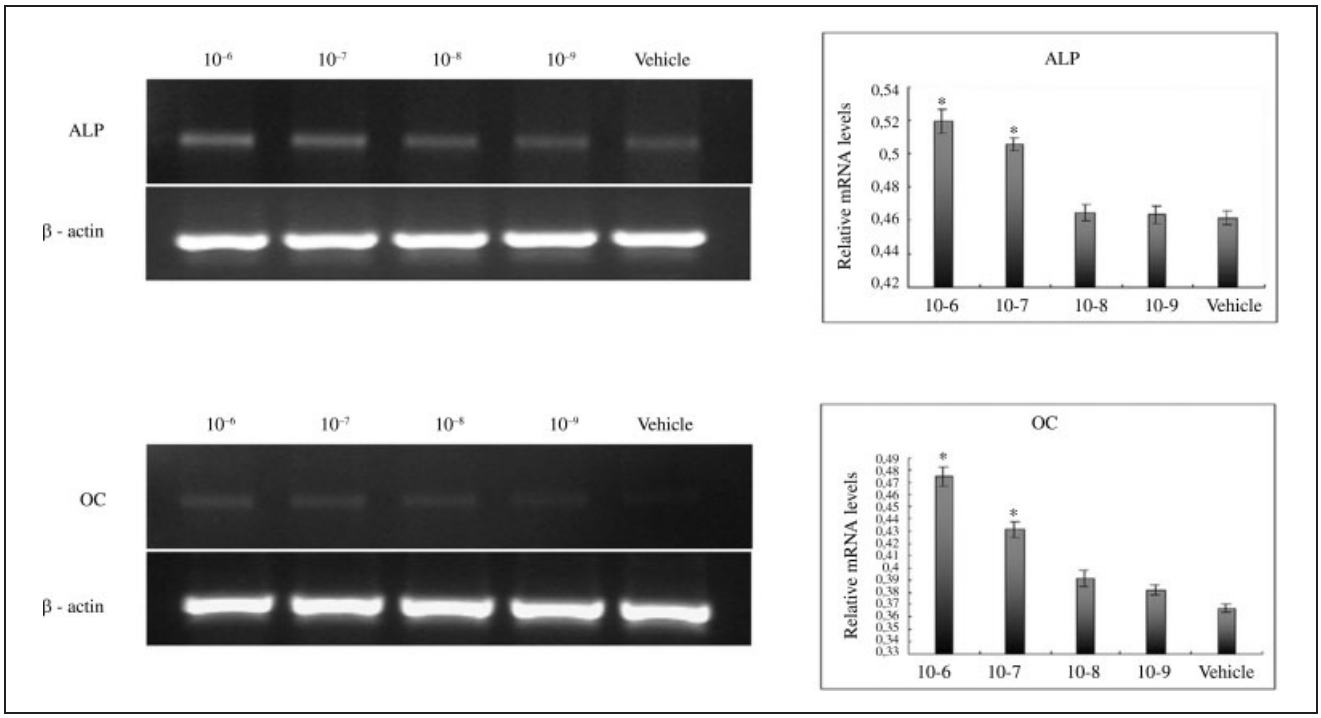

Fig. 3: Effect of simvastatin on alkaline phosphatase (ALP) and osteocalcin (OCN) mRNA expressions in aging rats MSCs.
RT-PCR for the detection of osteoblastic marker expression, ALP at 10 days and OC at 21 days after MSCs were induced in osteogenic medium with vehicle or simvastatin (10^{-6} to 10^{-9} M). Semiquantitative RT-PCR reactions were performed. Results were standardized using β-actin as a housekeeping gene and expressed as relative mRNA levels. Representative agarose gels are shown in the left panel with corresponding densitometric analysis in the right panel. * $P < 0.05$ (compared with vehicle control)

an early marker of osteoblastic differentiation, so we first analysed ALP staining and activity of MSCs on day 10. The number of positive cells in ALP staining was increased with the addition of simvastatin in a dose-dependent manner (Figs. 1A–D). The ALP activity of the MSCs was normalized on the basis of protein content per disk. As shown in Fig. 1F, the ALP activity of MSCs was also enhanced. We showed that simvastatin at 10^{-6} and 10^{-7} M significantly improved the ALP staining and activity compared to the vehicle group.

Osteoblast differentiation is characterized by mineralized nodule formation, so we examined the effects of simvastatin on the mineralization of MSCs by AR-S staining on day 28. As shown in Figs. 2A–D, the coloured blocks represent the positive staining. Simvastatin stimulated mineralized nodule formation in a dose-dependent manner, and the significant effects were observed at a concentration of 10^{-6} and 10^{-7} M simvastatin compared to the vehicle group. Simultaneously, we quantified stained mineral deposits by measurement of the absorbance at 405 nm (Fig. 2F). The results also revealed that simvastatin at 10^{-6} and 10^{-7} M stimulated mineralization of MSCs.

To further demonstrate the effect of simvastatin on osteoblast differentiation of MSCs from the aging rats, RT-PCR was done to detect the gene expression of osteoblast-specific markers, ALP and OC, on days 7 and 28. ALP expression was easily detectable on day 7. In contrast, OC expression, a marker for advanced osteoblastic differentiation, was not achieved until day 28. The expression of ALP and OC were notably increased in a dose-dependent manner with the addition of simvastatin, as compared to the vehicle group. Semi-quantitative analyses showed that the significant effects were presented at a concentration of 10^{-6} and 10^{-7} M simvastatin (Fig. 3).

2.2. Effect of simvastatin on adipogenic differentiation of MSCs

Confluent cells were further cultured in adipogenic medium consisting of Dex, insulin, IBMX, and indomethacin with simvastatin (10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9} M) and ethanol vehicle. Morphologic observations showed that adipocytes existed in clusters. Lipid droplets in the cytoplasm of adipocytes were detected on day 4 and reached a maximal accumulation between 2 and 3 weeks. We examined lipid droplet accumulation by oil Red O stain on day 18; a longer induction period would lead to adipocyte loss by detachment from the matrix. As shown in Fig. 4, addition of simvastatin remarkably reduced adipocyte differentiation of MSCs and less lipid droplets in a dose-dependent manner were seen as compared to the vehicle group. Cell counting confirmed that the significant effect existed in the 10^{-6} and 10^{-7} M simvastatin-treated groups.

The adipocytic phenotypes were examined by RT-PCR. LPL, an early marker for adipocytic differentiation, was tested on day 2 and PPARγ_2, a late marker for mature adipocytes, was detected on day 18. As shown in Fig. 5, simvastatin inhibited the gene expressions of LPL and PPARγ_2 in a dose-dependent manner, and the semi-quantitative analyses further confirmed a decrease of adipocyte differentiation. The significant effects also existed with 10^{-6} and 10^{-7}M simvastatin.

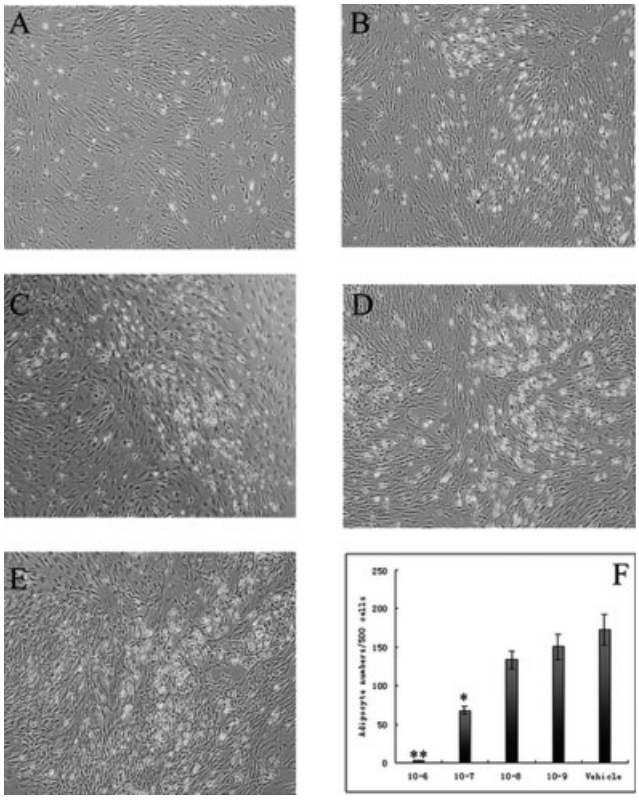

Fig. 4: Oil Red O staining for adipogenesis of MSCs affected by simvastatin.
MSCs were treated in adipoinductive medium with vehicle (E) or simvastatin (10^{-6} to 10^{-9} M; A–D) for 18 days. Adipocyte formation was monitored by Oil Red O staining. For quantification of adipogenesis, 500 cells/well were randomly examined under a microscope, and the number of adipocytes with Oil Red O-stained lipid droplets was counted (F). The data are expressed as the mean ± SEM of 4 determinations. * $P < 0.05$, ** $P < 0.01$ (compared with vehicle control)

3. Discussion

The vast majority of the literature reports MSCs residing in bone marrow have the ability to differentiate into multiple lineages, including adipocytic, chondrocytic, and osteoblastic lineages, and in some cases, towards a neural lineage (Hung et al. 2002; Sanchez-Ramos et al. 2000; Sanchez-Ramos 2002; Woodbury et al. 2002). It has been generally accepted that the reciprocal lineage determination between osteoblasts and adipocytes is a critical component in the regulatory pathways of osteogenesis (Gimble et al. 1996; Lecka-Czernik et al 1999). Aging can affect the bone marrow microenvironment in multiple ways and age-related changes are recognized by a decline in the "stemness" of cells which results in a decrease of osteogenesis and replacement of bone marrow with fat cells (Bellows et al. 2003; Moerman et al. 2004; Stenderup et al. 2003). Consistently, the increased lipid accumulation in bone marrow is reported in association with age-related bone loss. Additionally, gene expression profiling of MSCs from aging showed a decrease in genes involved in osteoblast differentiation with an increase in genes in adipocyte differentiation compared with juvenile rats (Akavia et al. 2006; Xiao et al. 2007). Therefore, enhancement of osteoblastogenesis and a concomitant inhibition of marrow adipocyte differentiation could provide a potential strategy for the treatment of age-related osteoporosis.

Statins, competitive inhibitors of HMG-CoA reductase, act at a rate-limiting step in cholesterol synthesis by strongly blocking conversion of HMG-CoA to mevalonate (Istvan and Deisenhofer 2001). Different statins have been reported to have various effects on bone, with lovastatin and pravastatin exhibiting the least effect, and simvastatin,

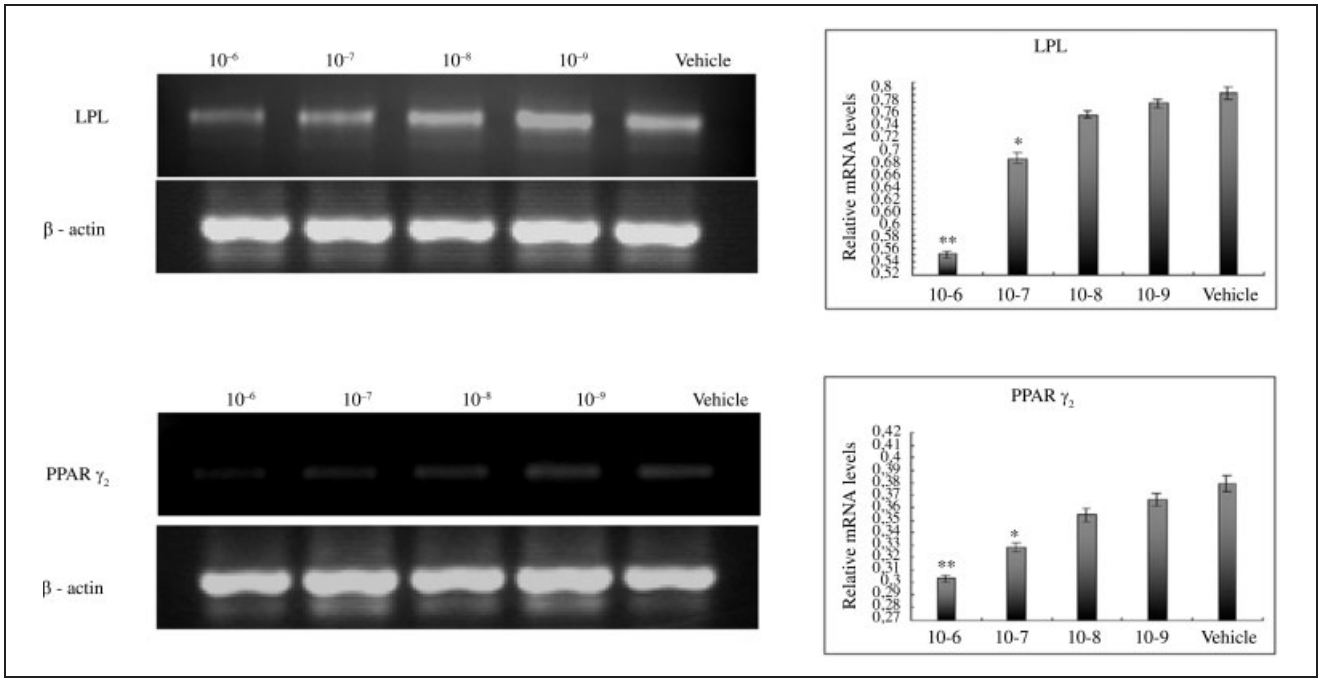

Fig. 5: Effect of simvastatin on lipoprotein lipase (LPL) and PPARγ_2 mRNA expression in aging rat MSCs.
RT-PCR for the detection of adipocyte marker expression; LPL at 2 days and PPARγ_2 at 18 days after MSCs were induced in adipogenic medium with vehicle or simvastatin (10^{-6} to 10^{-9} M). Semi-quantitative RT-PCR reactions were performed. Results were standardized using β-actin as a housekeeping gene and expressed as relative mRNA levels. Representative agarose gels are shown in the left panel with corresponding densitometric analysis in the right panel. * $P < 0.05$, ** $P < 0.01$ (compared with vehicle control)

atrovastatin, and cerivastatin exerting greater effects (Mundy 2001). Moreover, serivastatin has been removed from the market for its side effect on muscles and atrovastatin is inferior to simvastatin in the medical market. So, simvastatin was chosen from various HMG-CoA reductase inhibitors. Mundy et al first reported that simvastatin and lovastatin activate the BMP-2 promoter linked to a luciferase reporter gene in human osteoblast-like osteosarcoma (MG63) and murine osteoblastic (2T3) cells and stimulate *in vivo* bone formation in murine calvaria. Subsequently, Sugiyama et al. (2000) and Ohnaka et al. (2001) reported that simvastatin and mevastatin enhance the expression of BMP-2 mRNA and its protein in human osteosarcoma cells. More recently, Maeda et al. reported that simvastatin stimulates the expression of bone anabolic factors, such as vascular endothelial growth factor (VEGF) and bone morphogenetic protein-2 (BMP-2), and promotes osteoblast differentiation and mineralization in mouse calvarial osteoblastic cells (MC3T3-E1) and juvenile rat bone marrow cells. Li et al. (2003) and Song et al. (2003) found statins may inhibit adipogenic differentiation in D1 cells and young rat MSCs *in vitro*. According to the above studies, we reasoned that statins may enhance osteoblast differentiation and inhibite adipocyte differentiation. However, all these results were achieved by cell lines or MSCs from young rats, which overlooked MSCs from the aged more readily differentiate into adipocytes and less frequently into osteoblasts compared with the young. Therefore, these studies were not in agreement with age-related osteoporosis nor did they reflect the real role of statins in the differentiation of MSCs from the aged. So, we studied whether simvastatinin MSCs of aging rats had the same ability to decrease adipocyte formation and increase osteoblast differentiation. This is the first report about the effect of statins on the differentiation of MSCs in the aging rat.

MSCs from aging male SD rats (18 months old) were isolated and cultured. For osteoblast differentiation, although the precise physiologic role of ALP in bone is unknown, mature osteoblasts are characterized by high ALP activity, which makes it an early osteoblastic differentiation marker (Gori et al. 1999). OC, a late osteoblastic differentiation marker, is closely related to osteoblast maturation (Aubin et al. 1995). Mineralized nodule formation is a late stage of osteogenesis. Thus, we examined the staining and activity of ALP, the gene expression of ALP and OC, and mineralization. The results proved that simvastatin stimulated ALP staining and activity, enhanced the gene expressions of ALP and OC, and promoted the mineralization of the matrix in a concentration-dependent manner following the addition of simvastatin. Significant effects were observed at simvastatin concentrations of 10^{-6} and 10^{-7} M. Therefore it is suggested that simvastatin, for aging MSCs, possesses the same capability of stimulating osteoblastic differentiation as in the young. The OC gene was not expressed until day 28. We thought it may be related to a decrease in the osteogenic potential of the aged.

For adipocyte differentiation of aging MSCs, we tested Oil Red O staining and the gene expression of LPL and PPARγ_2. Oil Red O staining can identify lipid droplets in mature adipocytes. LPL is an early marker of adipocyte differentiation (Ahdjoudj et al. 2002) and PPARγ_2 is a late marker of adipogenesis (Knouff and Auwerx 2004). We found that simvastatin remarkably inhibited adipocyte differentiation of MSCs and reduced the levels of expression of mRNA for LPL and PPARγ_2. These results are consistent with previous observations (Phillips et al. 2001). The significant effects of simvastatin were also observed at concentrations of 10^{-6} and 10^{-7} M. However, we did not observe the same effect of 10^{-8} M simvastatin as that described previously by other authors. We believe that this discrepancy might result mainly from the different target cells. Because MSCs from the aged have a reinforcement in adipogenesis and a decline in osteogenesis, obtaining the same effect could require a larger concentration of

simvastatin. Additionally, we also found that if Dex, at a lower dose essential for osteoinduction (Cheng et al. 1994; Czock et al. 2005; Fried et al. 1996; Shur et al. 2001), was not added in the culture medium of osteoblastic differentiation, simvastatin could not independently induce MSCs into osteoblasts (data not shown), which coincided with the results achieved by Masato et al. (Sonobe et al. 2005). Simvastatin might just have the modest capability of osteogenesis, and a more significant effect on inhibiting adipogenesis. Nevertheless, the relationship between lipid-lowering and bone density-increasing has not been clarified, so we thought that this could be the reason that numerous contradictory results existed *in vivo* studies about statins.

In summary, we demonstrated that simvastatin inhibited adipocytic differentiation of MSCs from aging rats and promoted osteoblastic differentiation. If the *in vivo* relationship between lipid-lowering and bone formation will be further understood and new statins that are more specific and potent for bone metabolism could be developed, we believe that statins could become potential anabolic agents that enhance bone formation for patients with osteoporosis.

4. Experimental

4.1. Isolation and culture of aging rat MSCs

MSCs were cultured from aging male SD rats (18 months old). The bone marrow cells were isolated by flushing the femurs with 10 ml Dulbecco's modified essential medium (DMEM; Gibco^{TM}, invitrogen, NY,USA), supplemented with 10% heat-inactivated fetal calf serum (FCS) (JRH, Lenexa, Kansas, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin (HyClone). Clumps of bone marrow were gently minced with a pipette. The cells were incubated at 37 °C in 5% CO_2. Non-adherent cells were removed by replacing the medium after 7 days. Cells were grown for a further 7 days to sub-confluency, then washed with phosphate-buffered saline (PBS) and lifted by incubation with 0.25% trypsin/0.02% EDTA (Invitrogen, Carlsbad, CA, USA) for 5 min. Non-detached cells were discarded and the remaining cells were regarded as passage 1 of the MSCs culture.

4.2. Osteogenic and adipogenic differentiation

For osteoblast-like cell-induced cultures, MSCs were seeded at a density of 15000 cells/cm^2. Confluent MSCs were treated with osteogenic medium composed of dexamethasone (Dex; 10 nM), β-glycerophosphate (10 mM), and Vc ascorbic acid 2-phosphate (50 μg/mL; all from Sigma, St. Louis, MO, USA). For adipocyte-like cell-induced cultures, MSCs were seeded at a density of 30000 cells/cm^2. After confluence, MSCs were treated with adipogenetic medium composed of 3-isobutyl-1-methylxanthine (IBMX; 0.5 mM), Dex (1 μM), indomethacin (100 μM), and insulin (10 mM; all from Sigma). Simultaneously, cells were exposed to various doses of simvastatin (Calbiochem, EMD Bioscience, Sandiego, CA, USA) dissolved in 75% ethanol at 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9} M, and ethanol vehicle at the time periods indicated.

4.3. Alkaline phosphatase staining and activity assay

For osteoblast-like cell-induced cultures, the alkaline phosphatase (ALP) staining and activity assay was performed on day 10. After fixation with ethanol, the cultures were stained histochemically for ALP, as described in the manufacturer's instructions (Rainbow, Shanghai, China). The cells were exposed for 30 min at 20 °C to a solution of naphthol phosphate (0.05 mg/ml) in Tris buffer (0.08 M [pH 7.5]) containing fast red bb (1 mg/ml). The cultures were then washed under running tap water and photographed using a BH-2 Olympus microscope (TOKYO, Japan). For the level of ALP activity, MSCs were rinsed two times with PBS, followed by trypsinization and then scraped into ddH_2O. This was followed by three cycles of freezing and thawing. ALP activity was determined at 405 nm using p-nitrophenyl phosphate (pNPP; Sigma) as the substrate (Kasten et al. 2003). A 50 μl sample was mixed with 50 μl pNPP (1 mg/ml) in 1M diethanolamine buffer containing 0.5 mM $MgCl_2$ (pH 9.8) and incubated at 37 °C for 15 min on a bench shaker. The reaction was stopped by the addition of 25 μl of 3N NaOH per 100 μl of reaction mixture. Enzyme activity was quantified by absorbance measurements at 405 nm (Safire^2, TECAN). The total protein content was determined with the BCA method in aliquots of the same samples with the PIERCE protein assay kit (Rockford, IL, USA), read at 562 nm, and calculated according to a series of albumin (BSA) standards. ALP levels were normalized to the total protein content at the end of the experiment. All experiments were conducted in triplicate.

4.4. Assay of mineralized matrix formation

For osteoblast-like cell-induced cultures, an assay of mineralized matrix formation was performed at day 28. After removing the medium, cells were washed twice with PBS. The extent of mineralized matrix in the plates was determined by Alizarin Red S (AR-S) staining. Briefly, cells were fixed in 70% ethanol for 1 h at room temperature, washed with PBS, and stained with 40 mM AR-S (pH 4.2) for 10 min at room temperature. Next, the cell preparations were washed five times with deionized water and incubated in PBS for 15 min to eliminate non-specific staining. The stained matrix was photographed using an inverted BH-2 Olympus microscope. After that, AR-S staining was released from the cell matrix by incubation in 10% (w/v) cetylpyridinium chloride for 15 min. The amount of dye released was quantified by spectrophotometry at 562 nm. All experiments were conducted in triplicate.

4.5. Oil Red O staining

For adipocyte-like cell-induced cultures, Oil Red O staining was performed on day 18. The cells were rinsed once with PBS and then fixed in 10% formaldehyde for 10 min and then in 60% isopropanol for 1 min, stained with Oil Red O for 30 min, and rinsed briefly with 60% isopropanol. The cultures were then photographed using inverted BH-2 Olympus microscope. For quantification of adipogenesis, 500 cells/well were randomly examined under a microscope, and the number of adipocytes with Oil Red O-stained lipid droplets was counted. All experiments were performed in triplicate.

4.6. RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

After MSCs were treated in osteoblast-like cell-induced cultures for 10 and 28 days, and in adipocyte-like cell induced-cultures for 2 and 18 days, total RNA was isolated from MSCs using TRIZOL reagent (Invitrogen). Addition of chloroform to the thawed cell extract separated the RNA into an aqueous phase, which was recovered and precipitated with isopropanol. The RNA pellet was washed with 70% ethanol treated with the RNase inhibitor, diethyl pyrocarbonate (DEPC), and then solubilized in sterile DEPC water. Assessment of the concentration and quality of the total RNA samples were carried out by spectrophotometry. For osteogenic differentiation, the extracted RNA samples were analyzed by osteogenic markers as follows: ALP (day10) and osteocalcin (OC; day28). For adipogenic differentiation, the extracted RNA samples were analyzed by adipogenic markers as follows: lipoprotein lipase (LPL; day 2) and PPARγ_2 (day 18). β-actin was utilized as a housekeeping gene. Reverse transcription was carried out according to the manufacturer's instructions using reverse transcriptase (Invitrogen), random hexamers and dNTP. Template DNA was then used in gene-specific PCR for ALP, OCN,

Table: Primer sequences and cycle conditions used for RT-PCR

Gene	Primer sequence (forward/reverse)	T annealing (°)	Cycles
ALP (418bp)	5′-CTACTTGTGTGGCGTGAAGG-3 ′ 5′-AATGCTGATGAGGTCCAGG-3′	54	30
OC (249bp)	5′-GCAGAAGGGAAGGGAC-3′ 5′-GAACAGAGGCACAGGAT-3′	54	30
LPL (208bp)	5′-ATCCAGCTGGGCCTAACTTT-3′ 5′-AATGGCTTCTCCAATGTTGC-3′	52	30
PPARγ_2 (248bp)	5′-TATGGAGCCTAAGTTTGAGTT-3′ 5′-CAATCTGCCTGAGGTCTG-3′	54	30
β-actin (230bp)	5′-CCGTAAAGACCTCTATGCCAACA-3′ 5′-CGGACTCATCGTACTCCTGCT-3′	54	28

LPL, PPARγ_2, and β-actin, details of primers and reaction temperatures are listed in the Table. The PCR products were detected by 1.0% agarose gel electrophoresis and photographed. Analysis of RNA levels was performed by normalization to the housekeeping gene (β-actin) using densitometry (BioRad, Hercules, CA, USA).

4.7. Statistical analysis

All values are expressed as the means ± standard deviations. Differences between treated and untreated groups were assessed by Student's t-test. Multiple comparisons were evaluated by ANOVA, followed by Scheffe's F-test. Statistical analysis was performed with the software, SPSS11.5. A P value < 0.05 was considered to indicate statistical significance.

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