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Lactone form 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors (statins) stimulate the osteoblastic differentiation of mouse periodontal ligament cells via the ERK pathway

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Background and Objective: Recent studies reported that the lactone forms of 3-hydroxy- 3-methylglutaryl-coenzyme A reductase inhibitors, which are also known as statins, have a bone stimulatory effect. However, there are few reports on the effect of statins on periodontal ligament cells. This study examined the statin-induced osteoblastic differentiation of mouse periodontal ligament cells as well as its mechanism.

Material and Methods: Mouse periodontal ligament cells were cultured with lovastatin or simvastatin, and their viability was measured. The levels of alkaline phosphatase (ALP), osteocalcin, bone sialoprotein and bone morphogenetic protein-2 mRNA expression were evaluated by RT-PCR. The osteoblastic differentiation was characterized by the ALP activity and Alizarin Red-S staining for calcium deposition. The activity of the osteocalcin gene (*OG2*) and synthetic osteoblast-specific elements (6× *OSE*) promoter with statins was also measured using a luciferase assay. For the signal mechanism of statins, the ERK1/2 MAPK activity was determined by western blot analysis.

Results: A statin treatment at concentrations < 1 μM did not affect the cell viability. Lovastatin or simvastatin at 0.1 μM increased the levels of ALP, osteocalcin, bone sialoprotein and bone morphogenetic protein-2 mRNA in mouse periodontal ligament cells. In addition, the ALP activity, mineralized nodule formation and *OG2* and *OSE* promoter activity were higher in the lovastatin- or simvastatin-treated cells than the control cells. Western blot analysis confirmed that the statins stimulated the phosphorylation of ERK1/2.

Conclusion: Lovastatin and simvastatin may stimulate the osteoblastic differentiation of periodontal ligament cells via the ERK1/2 pathway. This suggests that the statins may be useful for regenerating periodontal hard tissue.

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The ultimate goal of periodontal regeneration therapy is to regenerate lost periodontal tissues. The periodontium is a highly specialized organ, consisting of cementum, periodontal ligament, gingiva and alveolar bone. Periodontal ligament fibroblasts and gingival fibroblasts are the major cells in the periodontal ligament and gingiva, respectively, whereas osteoblasts are the primary cells responsible for alveolar bone formation (1).

The periodontal ligament is a highly vascularized and cellularized connective tissue that attaches the root of the tooth to the surrounding alveolar bone (2,3). The cells of the periodontal ligament exhibit some of the characteristics of osteoblasts, in that they support new bone formation *in vivo*. Moreover, periodontal ligament cells cultured with 1α,25-dihydroxyvitamin D_3 produces RANKL (4). These findings suggest that the periodontal ligament is involved in regulating the alveolar bone metabolism.

Statins inhibit 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, which is the rate-limiting enzyme for cholesterol biosynthesis, and are used widely as cholesterol-lowering drugs. Mundy *et al.* (5) reported that statins enhance the expression of bone morphogenetic protein-2 mRNA in osteogenic cells *in vitro* and stimulate bone formation when injected subcutaneously over murine calvaria. Some *in vitro* studies also suggested that statins promote the differentiation of osteoblastic cells (6–8). In addition, the topical application of statins in the bone microenvironment has been shown to stimulate new bone formation (9,10). Sugiyama *et al.* attempted a subcutaneous injection of statins to avoid the accumulation of statins in the liver and to deliver them to the peripheral tissue. They suggested that the subcutaneous administration of simvastatin stimulate ectopic osteoinduction by recombinant human bone morphogenetic protein-2 by reducing the level of bone turnover (11). Lipophilic statins, namely lovastatin, fluvastatin, simvastatin and mevastatin, specifically activate the bone morphogenetic protein-2 gene promoter (5). The transient exposure of bone cell cultures to lipophilic statins is sufficient to initiate the cascade resulting in osteoblast differentiation, most probably due to the local production of bone morphogenetic protein-2.

This osteogenic effect of statins might be of interest in regenerating periodontal defects, particularly hard tissue regeneration. Most studies have examined the effects of statins on osteoblast differentiation using animal or human immortalized cell lines and with primary human osteoblasts. The main concern of ours was whether statins have an additional effect on osteogenesis by periodontal ligament cells.

This study examined the effects of lovastatin or simvastatin (lactone form of statins) on osteoblastic differentiation and mineralization as well as the molecular mechanism for the statin-induced osteoblastic differentiation of mouse periodontal ligament cells.

Material and methods

Cell cultures

Immortalized mouse periodontal ligament cells (12) were a generous gift from Dr M. J. Somerman (University of Washington, Seattle, WA, USA) and K. Kirkwood (Medical University of South Carolina, Charleston, SC, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Gibco BRL) and antibiotics (100 U/mL of penicillin and 100 μg/mL of streptomycin) at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. To induce osteoblastic differentiation, the cells were incubated in a differentiation medium supplemented with 2% FBS, antibiotics, ascorbic acid (50 μg/mL) and β-glycerophosphate (10 mM; Sigma Chemical Co. St Louis, MO, USA) for up to 21 d in the presence or absence of lovastatin and simvastatin (Wako Pure Chemical Inc., Osaka, Japan). Lovastatin and simvastatin were dissolved separately in 75% ethanol at concentrations ranging from 0.1 to 1 mM. The ethanol vehicle was used as a control. The statins were added to the cell culture containing the differentiation medium, and the medium was changed every other day. U0126 (10 mM; Cell Signaling, Danvers, MA, USA), which is inhibitor of both MEK1 (mitogen-activated ERK kinase 1) and MEK2, was stored in ethanol at −80°C at a concentration of 10 mM until needed.

Cytotoxicity evaluation

The mouse periodontal ligament cells were seeded in 96-well culture plates at a density of 1.5×10^4 cells per well and pre-incubated for 24 h in a growth medium, DMEM containing 10% FBS and antibiotics (100 U/mL of penicillin and 100 μg/mL of streptomycin). The cultures were exposed to either lovastatin or simvastatin at concentrations ranging from 0.01 to 1 μM for up to 72 h in a differentiation medium. The cell viability was examined using an XTT assay (Cell Proliferation kit II; Roche Applied Science, Indianapolis, IN, USA) according to the manufacturer's protocol.

RT-PCR analysis

The mouse periodontal ligament cells were seeded in six-well culture plates at a density of 2×10^5 cells per well and pre-incubated in a growth medium for 24 h. The culture medium was changed to a differentiation medium, and the cells were exposed to either 0.1 μM lovastatin or 0.1 μM simvastatin for 12–72 h. The untreated mouse periodontal ligament cells were used as a control. The total RNA was isolated from the culture using a TRIzol® reagent (Invitrogen Life Technology, Carlsbad, CA, USA) according to the manufacturer's instructions. The cDNA was synthesized using the random primer and reverse transcriptase (Invitrogen Life Technology) from the extracted total RNA. Subsequent amplification was performed as follows: initial denaturation at 95°C for 30 s followed by 27–30 cycles at 95°C for 15 s, annealing at a temperature optimized for each primer pair for 30 s, and 72°C for 30 s, and extension at 72°C for 7 min. Table 1 lists the primer sequences used for PCR amplification

Table 1. Sequences of the primers used for RT-PCR

Gene	Primer sequences (5′–3′)	Annealing temperature (°C)	Product size (bp)	References
Alkaline phosphatase	Forward: GATCATTCCCACGTTTTCAC Reverse: TGCGGGCTTGTGGGACCTGC	58	290	NM 201007
Osteocalcin	Forward: CTCCTGAGTCTGACAAAGCCTT Reverse: GCTGTGACATCCATTACTTGC	58	380	NM 207225
Bone sialoprotein	Forward: GAGGGCAGAGGAAATACTCAAT Reverse: ATTCAAAGCCAAGTTCAGAGATGTAAA	55	441	NW 002198
Bone morphogenetic protein -2	Forward: CTCCTGAGTCTGACAAAGCCTT Reverse: GCTGTGACATCCATTACTTGC	66	1200	NM 001020
β-Actin	Forward: TCATGAAGTGTGACGTTGACATCCGT Reverse: CCTAGAAGCATTTGCGGTGCACGATG	55	310	NM 007393

based on the published nucleotide sequence. All the primers were synthesized by Bioneer Co. (Taejeon, Korea). Although the results obtained by RT-PCR are not quantitative by definition, PCR analysis was performed within 30 cycles with relatively high linearity using the primer for the housekeeping gene β-actin. Each PCR product was loaded in 1.5% agarose gels by electrophoresis, and visualized by ethidium bromide staining. The band intensity was quantified by image analysis of the gel images using image software (Scion Image, Beta 4.0.3; Scion Corp., Frederick, MD, USA; http://www.scioncorp.com).

Measurement of alkaline phosphatase (ALP) activity

The mouse periodontal ligament cells were cultured in 24-well plates at a density of 4×10^4 cells per well with 0.01, 0.1 or 1 μM lovastatin or simvastatin in the differentiation medium for 6 d. For measurement of ALP activity, the cells were lysed and sonicated in 10 mM Tris–HCl (pH 7.5) containing 0.2% NP40 and 2 mM phenylmethylsulphonyl fluoride (Sigma Chemical Co.) and then centrifuged at 4500*g* at 4°C for 10 min. The supernatants were reacted with the ALP assay mixtures containing 0.1 M 2-amino-2-methyl-1-propanol, 1 mM $MgCl_2$, 8 mM *p*-nitrophenyl phosphate disodium (Sigma Chemical Co.) for 5 min at 37°C. The reaction was quenched by adding 0.1 N NaOH, and the absorbance of the resulting color measured at 405 nm using *p*-nitrophenol as a standard. The amount of double-stranded DNA in the cell lysate was measured using a Picogreen® dsDNA quantitation kit (Molecular Probes, Inc., Eugene, OR, USA) with a spectrofluorometer (PerkinElmer LS55; Norwalk, CT, USA excitation 480 nm, emission 520 nm) according to the manufacturer's protocol. The ALP activity was normalized to the total DNA content.

Alizarin Red staining for mineralized extracellular matrix

The mouse periodontal ligament cells were cultured in 24-well plates at a density of 4×10^4 cells per well with either 0.1 μM lovastatin or 0.1 μM simvastatin in the differentiation medium for 21 d. The culture plates were then rinsed with phosphate-buffered saline, and fixed with 70% ethanol for 1 h. After washing, the fixed cells were treated with a 40 mM Alizarin Red-S stain (Sigma Chemical Co.) solution for 10 min at pH 4.2 to stain the calcium deposits. The plates were washed five times with sterile distilled water, and then rinsed with phosphate-buffered saline. The stained culture plates were photographed, which was followed by a quantitative eluting procedure using 10% (w/v) cetylpyridinium chloride in 10 mM sodium phosphate, pH 7.0, for 15 min at room temperature. Aliquots of these Alizarin Red-S extracts were diluted in a 10% cetylpyridinium chloride solution, and the Alizarin Red-S concentration was determined by measuring the absorbance at 540 nm on a multiplate reader spectrophotometer (Bio-Tek Instruments Inc., Winooski, VT, USA) using an Alizarin Red-S standard curve in the same solution.

Transfection and luciferase assay

The reporter constructs, 1.3 kb osteocalcin gene (*OG2*)-Luc and six-copy osteoblast specific element (6× *OSE*)-Luc, were kindly provided by Dr Renny Franceschi (University of Michigan School of Dentistry, Ann Arbor, MI, USA). The mouse periodontal ligament cells were transfected with the indicated report plasmids and the Cytomegalovirus β-galactosidase (β-Gal) expression vector as an internal control plasmid using FuGENE 6 (Roche Applied Science). The total amount of DNA in each transfection was adjusted by adding the appropriate amounts of pcDNA3 control vector. Approximately 24 h after transfection, the culture medium was changed to serum-free medium, and the cells were exposed to 0.01 or 0.1 μM lovastatin or simvastatin for 24 h. The cells were then lysed and assayed using the Dual Luciferase Reporter Assay System (Promega, Madison, WI, USA). The luciferase activity was normalized to the β-Gal activity.

Western blotting

The mouse periodontal ligament cells were serum-deprived for 24 h and plated at 2×10^4 cells/cm^2 in 35 mm dishes with a serum-free differentiation medium containing 0.1 μM of lovastatin or simvastatin for 5–60 min, with or without a pretreatment with U0126 for 2 h in serum-free differentiation medium. U0126 (MEK1/2 inhibitor) in ethanol was added to the incubation buffer at the onset of stimulated reperfusion, at the final concentration of 10 μM in the incubation

buffer. The total cells were harvested in a lysis buffer (Cell Signaling Technology, Beverly, MA, USA), centrifuged at 12,000*g* for 15 min at 4°C, and the supernatants stored at −80°C. Quantification of the total protein was determined using a BCA protein assay reagent (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts (30 μg) of the protein from each cell lysate were separated on a 10% sodium lauryl sulfate–polyacrylamide gel electrophoresis gel and transferred to a Polyvinylidene fluoride (PVDF) membrane (Millipore Corp., MA, Bedford, USA). After blocking in 5% skim milk in Tris-buffered saline (TBS) with 0.1% Tween-20 (TBS-T), the membrane was incubated with the specific primary antibodies to phospho-extracellular-signal-regulated kinases (ERK), ERK or β-actin (Cell Signaling), diluted 1:1000 in TBS-T containing 5% skim milk overnight at 4°C. The membranes were washed with TBS-T and incubated with a 1:3000 dilution of horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G in TBS-T for 2 h at room temperature. After three washes with TBS-T, the signals were determined by enhanced chemiluminescence according to the manufacturer's instructions (Amersham Biosciences, Piscataway, NJ, USA). The signal was quantified by image analysis using SCION IMAGE software.

Statistical analysis

Each experiment was repeated at least twice. Unless indicated otherwise, the experimental data are expressed as the means ± SD of triplicate independent samples. Student's unpaired *t*-test and ANOVA with a Duncan grouping was used to evaluate the statistical differences between the groups. A p-value < 0.05 was considered significant.

Results

Statin cytotoxicity of mouse periodontal ligament cells

The cytotoxicity of the statins was assessed using an XTT assay to examine the sensitivity of the mouse periodontal ligament cells to different concentrations of lovastatin and simvastatin (ranging from 0.01 to 1 μM) applied for 24–72 h. Lovastatin and simvastatin had a slightly cytotoxic effect at a concentration of 1 μM (Fig. 1). Therefore, a statin concentration of 0.1 μM was used for further experiments.

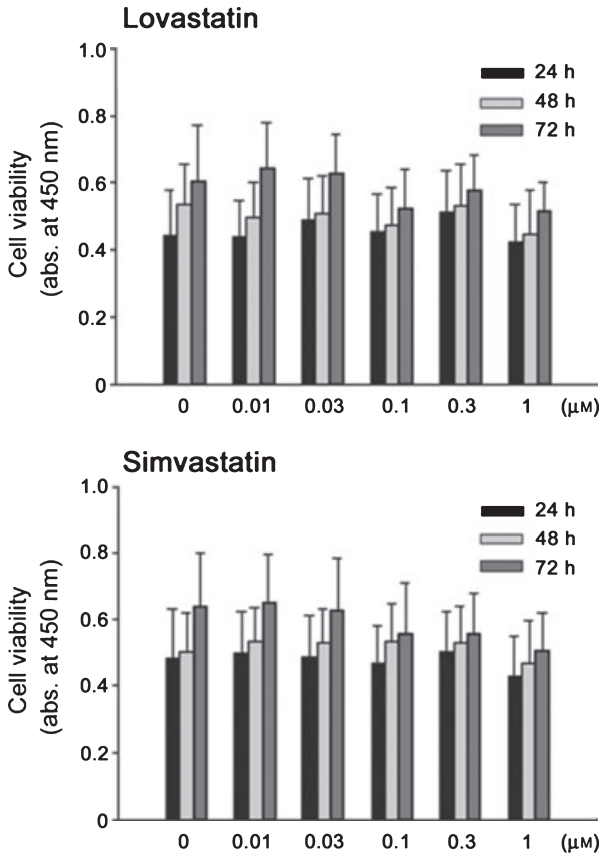

Fig. 1. Cytotoxic effects of lovastatin and simvastatin on mouse periodontal ligament cells. The mouse periodontal ligament cells were seeded in 96-well culture plates at a density of 1.5×10^4 cells per well and pre-incubated in growth medium, DMEM containing 10% FBS and antibiotics, for 24 h. The culture medium was changed to a differentiation medium containing ascorbic acid (50 μg/mL) and β-glycerophosphate (10 mM), and the cells were exposed to lovastatin and simvastatin at concentrations of 0.01–1 μM for 24–72 h. The cell viability was measured using an XTT assay.

Lovastatin and simvastatin stimulated the osteoblast-related gene expression in mouse periodontal ligament cells

The level of osteoblast-specific mRNA expression in mouse periodontal ligament cells was evaluated by RT-PCR to determine whether lovastatin and simvastatin affect osteoblast differentiation and bone formation. In the control mouse periodontal ligament cells incubated in differentiation medium, the levels of ALP, osteocalcin and bone sialoprotein mRNA were very low and increased slightly in a time-dependent manner, except for bone morphogenetic protein-2 mRNA, which was at an almost undetectable level for up to 72 h. When the mouse periodontal ligament cell cultures were treated with 0.1 μM lovastatin or simvastatin, the levels of ALP, osteocalcin, bone sialoprotein and bone morphogenetic protein-2 mRNA significantly increased after 24 h in a time-dependent manner (Fig. 2A). Compared with the lovastatin treatment, the simvastatin treatment resulted in faster and higher bone sialoprotein and bone morphogenetic protein-2 expression, but no increase in bone morphogenetic protein-2 expression was observed before 12 h exposure.

Compared with the control cultures, ALP expression was increased

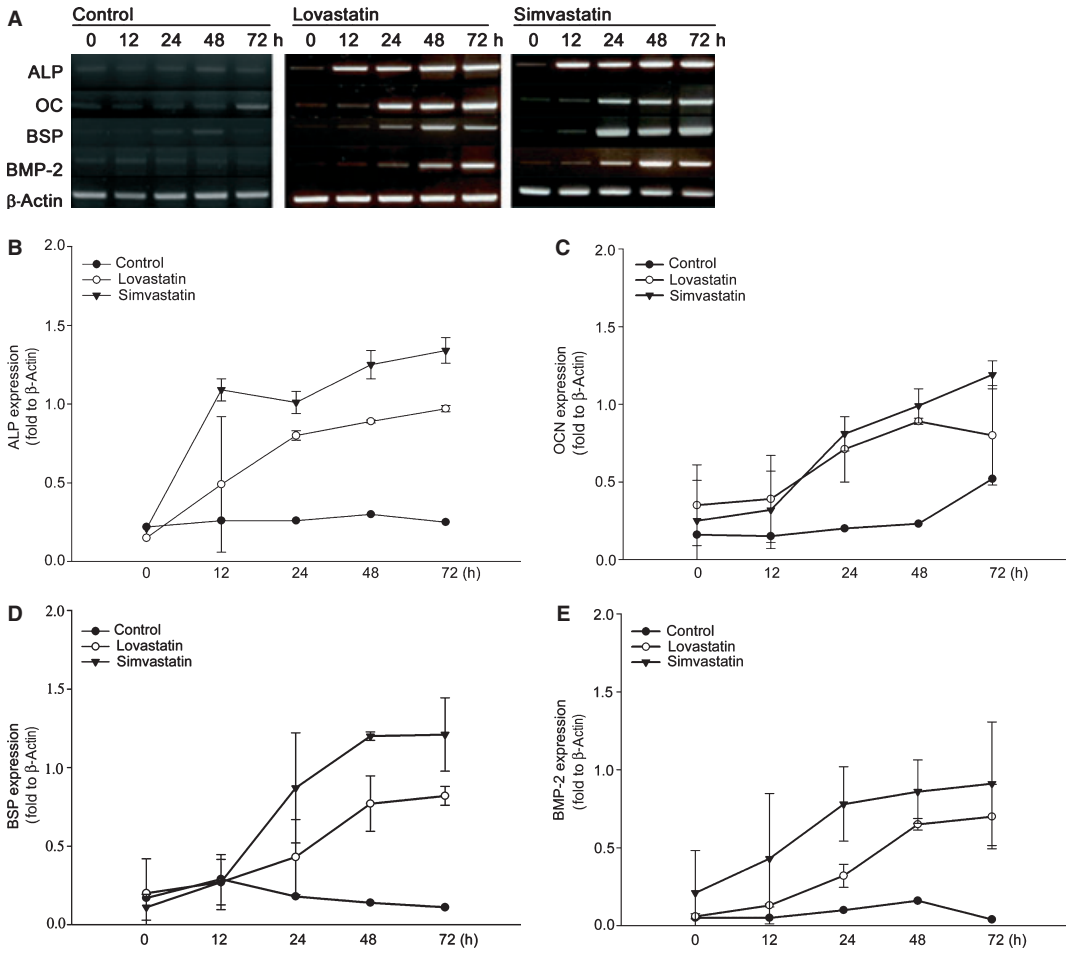

Fig. 2. Effects of lovastatin and simvastatin on osteoblast-related gene expression in mouse periodontal ligament cells. The mouse periodontal ligament cells were seeded in six-well culture plates at a density of 2×10^5 cells per well and pre-incubated in growth medium. After 24 h, the culture medium was changed to a differentiation medium, DMEM containing 2% FBS, ascorbic acid (50 μg/mL) and β-glycerophosphate (10 mM), and the cells were exposed to 0.1 μM lovastatin or simvastatin for 12–72 h. The untreated mouse periodontal ligament cells were used as a control. The total RNA was isolated and used for RT-PCR analysis with the indicated primers (A). ALP (B), osteocalcin (OCN; C), bone sialoprotein (BSP; D), and bone morphogenetic protein-2 (BMP-2; E) gene expression levels were plotted as multiples of the β-actin level. The data are expressed as the means ± SD of two independent experiments.

three- and fourfold by the lovastatin and simvastatin treatment, respectively, after 12 h exposure, and remained relatively constant thereafter (Fig. 2B). Osteocalcin expression was increased fourfold after 24 h but only twofold after 72 h with both statin treatments (Fig. 2C). Bone sialoprotein expression after simvastatin exposure was increased four- and 12-fold after 24 and 72 h, respectively. In contrast, bone sialoprotein expression was increased two- and eightfold after 24 and 72 h of the lovastatin treatment, respectively (Fig. 2D). Lovastatin exposure resulted in a fivefold increase in bone morphogenetic protein expression compared with the control cultures after 48 h, which increased to 20-fold after 72 h, whereas simvastatin exposure resulted in a 10-fold increase compared with the control cultures after 12 h and a >25-fold increase after 72 h (Fig. 2E).

Lovastatin and simvastatin stimulated osteoblastic differentiation of mouse periodontal ligament cells and intercellular matrix mineralization

The total cellular ALP activity in the lysate was measured to determine the effect of statins on the osteoblastic differentiation of mouse periodontal ligament cells. After 6 d culture, lovastatin and simvastatin led to an approximately 2.3- and 2.0-fold increase in ALP activity, respectively, compared with the control cultures. The effect reached a maximum at 0.1 μM, and decreased significantly at 1 μM simvastatin (Fig. 3A).

The effects of statins on the mineralized nodule formation *in vitro* were examined by culturing the mouse periodontal ligament cells in a differentiation medium containing either lovastatin or simvastatin at 0.1 μM. After 21 d culture, mineralized nodule formation was assessed by Alizarin Red-S staining. As shown in Fig. 3B, 0.1 μM lovastatin and simvastatin stimulated mineralization of the extracellular matrix (the nodule formation) by equal amounts in the mouse periodontal ligament cells (Fig. 3B).

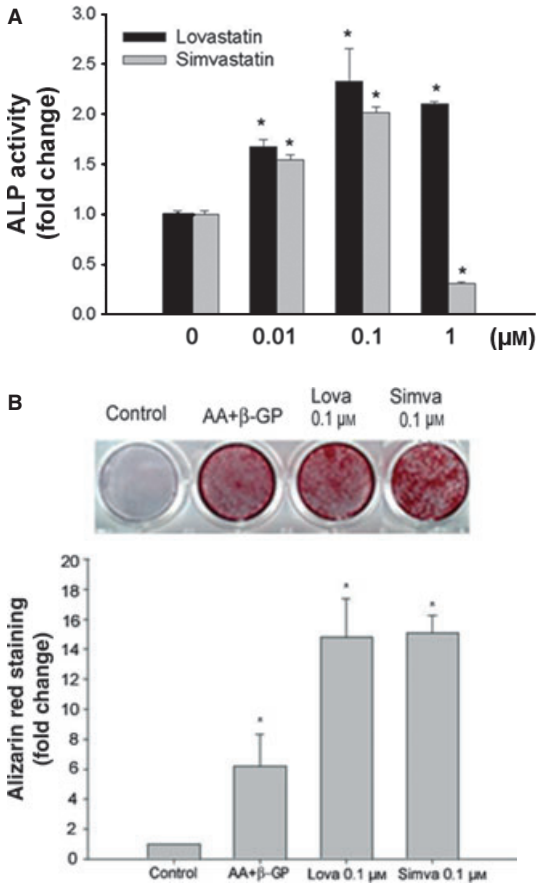

Fig. 3. Effect of lovastatin and simvastatin on osteoblastic differentiation and matrix mineralization in mouse periodontal ligament cells. (A) ALP activity. The mouse periodontal ligament cells were cultured in 24-well plates at a density of 4×10^4 cells per well with 0.01 or 0.1 μM lovastatin or simvastatin in a differentitation medium for 6 d. The cellular ALP activity was measured from the cell lysates. (B) Matrix mineralization. The mouse periodontal ligament cells were cultured with 0.1 μM lovastatin or simvastatin in the diffentiation medium. After 21 d, the mineral deposits on the cell cultures were stained with Alizarin Red-S. To quantify the level of mineralization, the stained Alizarin Red-S was eluted from the cell cultures with 10% cetylpyridinium chloride, and the dye concentration was measured using a spectrophotometer. The data are expressed as the means ± SD of triplicate samples (*$p < 0.01$). (Control, cell culture in DMEM supplemented with 2% FBS and antibiotics; AA + β-GP, cell culture in a differentiation medium of DMEM supplemented with 2% FBS and antibiotics, AA (ascorbic acid) and β-GP (β-glycerophosphate)

Lovastatin and simvastatin stimulated the transactivation of *OG2* or *OSE* promoter

Two reporter constructs fused with luciferase (*OG2*-Luc and 6× *OSE*-Luc) were used to examine the effects of lovastatin or simvastatin on the transcriptional activity of osteocalcin or osteoblast specific marker genes in mouse periodontal ligament cells. Lovastatin or simvastatin at 0.01 and 0.1 μM increased the activities of the *OG2*-Luc and 6× *OSE*-Luc reporter genes by more than four- and 40-fold, respectively (Fig. 4).

Lovastatin and simvastatin enhanced ERK phosphorylation in mouse periodontal ligament cells

The level of ERK expression in mouse periodontal ligament cells was examined by western blotting to determine the signaling pathway for how the statins promote osteoblastic differentiation. The accumulation of phospho-ERK was evaluated in the lysate of the mouse periodontal ligament cells after being treated with 0.1 μM lovastatin or simvastatin with and without pretreatment with a U0126 (inhibitor of both MEK1 and MEK2; Fig. 5A,B). In the untreated control cultures, the level of ERK phosphorylation was not pronounced, and increased slightly after 30 min incubation. A pretreatment with U0126 did not cause any significant changes. The effect of the statins on the phosphorylation of ERK1/2 was rapid; 0.1 μM lovastatin or simvastatin caused the accumulation of phospho-ERK1/2 in mouse periodontal ligament cells within 5 min of treatment. In the mouse periodontal ligament cells treated with 10 μM U0126, the effects of statin-induced ERK phosphorylation were blocked completely after lovastatin exposure, but only partly after simvastatin exposure (Fig. 5C).

To address whether the ERK pathway affects osteogenic differentiation in periodontal ligament cells, we did ALP staining assay using BCIP/NBT liquid substrate system (Sigma Chemical Co.) in the mouse periodontal ligament cells treated with 0.1 μM lovastatin or simvastatin in a differentitation medium with or without 10 μM U0126 (MEK1/2 inhibitor) for 7 d. The result showed that the intensity of positive staining decreased with U0126 treatment by 40–50% in cells treated with either statin (Fig. 6).

Discussion

For periodontal tissue reconstruction, it is essential that multipotent progenitor cells or putative stem cells be present in the periodontal ligament. Several studies suggested that periodontal ligament fibroblastic cells and bone marrow mesenchymal cells have common biological characteristics (13,14), and periodontal ligament cells exhibit similar *in vitro* phenotypic characteristics to osteoblast-like cells (15,16).

The HMG-CoA reductase inhibitors, the so-called statins, have been introduced as the most potent cholesterol-lowering agents and are potent stimulators of osteoblast differentiation and bone formation in osteoblasts (5,17). This study demonstated

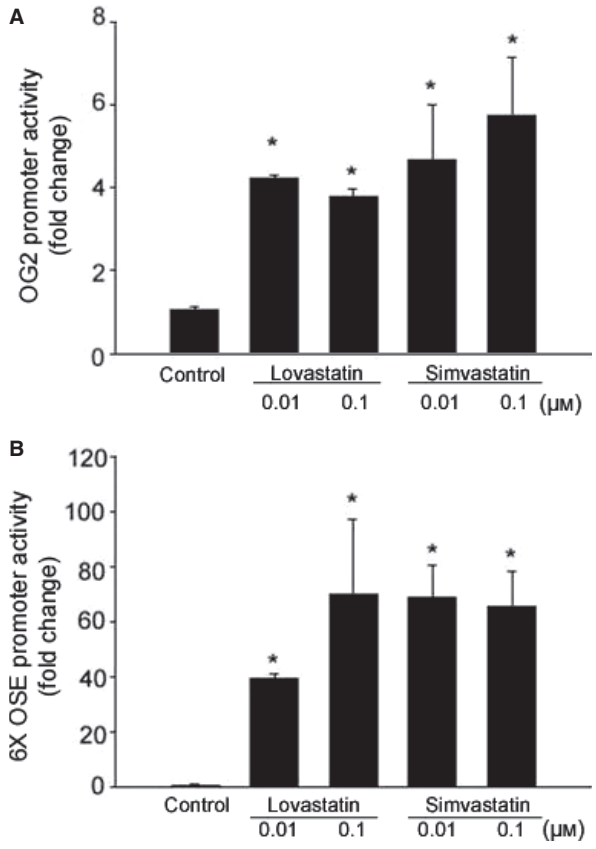

Fig. 4. Effect of lovastatin and simvastatin on the transcriptional activity of the osteocalcin gene (*OG2*) and osteoblast specific element (*OSE*) promoter genes. The mouse periodontal ligament cells were transfected with *OG2*-Luc or 6× *OSE*-Luc plasmid. Cytomegalovirus β-galactosidase was also added as an internal control plasmid. After exposing the mouse periodontal ligament-Luc cells to 0.01 and 0.1 μM of lovastatin or simvastatin for 24 h, the cells were lysed, and the luciferase activity was measured and normalized to the β-galactosidase activity. The data are expressed as the means ± SD of triplicate samples (*$p < 0.01$).

that statins, particularly the lactone forms, also have stimulatory effects on the osteoblastic differentiation of mouse periodontal ligament cells via the ERK pathway. In this study, well-defined healthy mouse periodontal ligament cells, which are the immortalized murine cell population isolated from the root surface, were used (12) because human periodontal ligament cells are sensitive to a range of noxious stimuli. The osteoblastic transdifferentiation traits of the mouse periodontal ligament cells were reconfirmed by the observation that bone morphogenetic protein-2 (100 ng/mL) increased the ALP activity in the cells (data not shown).

The process of bone formation consists of the following three main developmental stages: proliferation, development of the extracellular matrix and mineralization (18). Therefore, the ideal drug for periodontal bone regeneration requires minimal inhibition of periodontal ligament cell proliferation with the maximal stimulation of osteoblastic differentiation. In this study, 0.1 or 0.3 μM of simvastatin or lovastatin induced the osteoblastic differentiation of mouse periodontal ligament cells without inhibiting cell proliferation.

Mundy *et al.* (5) first reported that simvastatin and lovastatin stimulated *in vivo* bone formation when injected subcutaneously over the calvaria of mice, which resulted from increases in bone morphogenetic protein-2 expression in osteoblast cells via the activation of a gene promoter. The osteogenic effect of statins was inhibited by noggin, an endogenous inhibitor of bone morphogenetic proteins (19), and the statin-mediated activation of the bone morphogenetic protein-2 promoter was prevented by the addition of mevalonate, a downstream metabolite of HMG-CoA reductase for cholestereol biosynthesis. Therefore, the use of statins is considered to be a good strategy for controlling patients with hyperlipidemia and osteoporosis (17). Bone morphogenetic protein-2 production may also play an important role in periodontal tissue regeneration and healing (13,14). In the present study, 0.1 μM lovastatin and simvastatin induced bone morphogenetic protein-2 mRNA expression in mouse periodontal ligament cells with increases in ALP, osteocalcin and bone sialoprotein mRNA expression, as observed in osteoblast cells. In addition, the statins enhanced the ALP enzyme activity of mouse periodontal ligament cells and mineral deposition in the mouse periodontal ligament cell culture layers, suggesting that statins may also stimulate *in vivo* bone formation in the periodontal ligament.

The accumulation of extracellular matrix protein, especially highly calcium-binding osteocalcin and bone sialoprotein, is essential for bone formation. These RT-PCR results show that statins stimulated the expression of osteocalcin and bone sialoprotein mRNA in mouse periodontal ligament cells (Fig. 2). In addition, this study examined the effect of lovastatin and simvastatin on the transcriptional activity of the genes in mouse periodontal ligament cells using two reporter constructs fused with luciferase: *OG2*-Luc, a mouse osteocalcin promoter; and *OSE*-Luc, an osteoblast specific *cis*-acting element promoter. As a result, both statins at 0.1 μM increased the activities of the two reporter genes (Fig. 4). These results suggest that lovastatin and simvastatin positively regulate the transcriptional activity of the osteoblast marker genes in mouse periodontal ligament cells, as in osteoblast cells. Considering that bone morphogenetic protein-2 promotes the transactivation of the matrix protein genes, such as ALP, bone sialoprotein and osteocalcin, through the Smad1/5/8 pathway, the osteogenic effects of statin in mouse periodontal ligament cells might be related to the

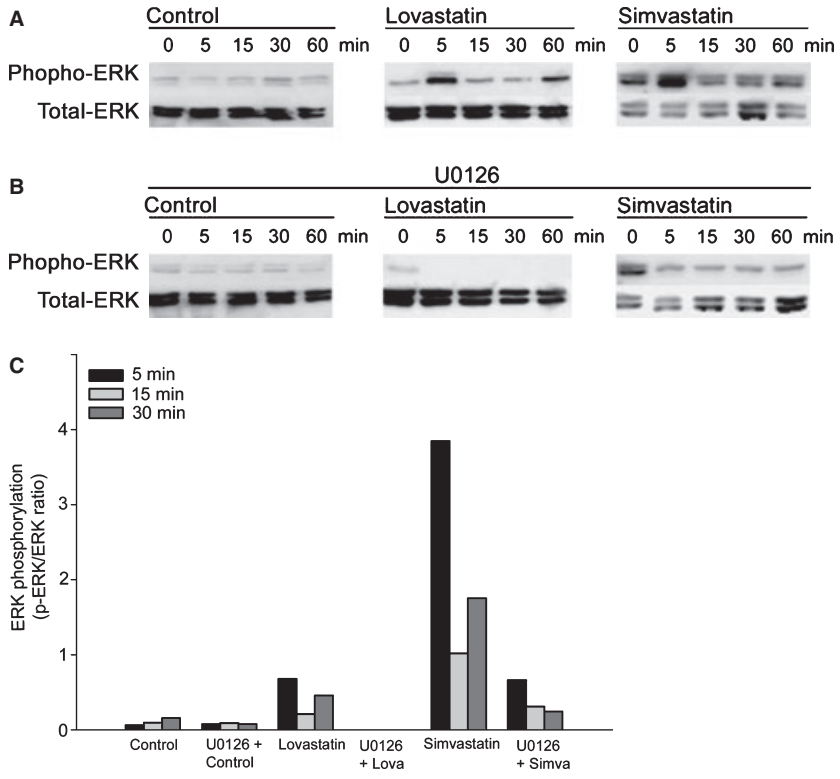

Fig. 5. Lovastatin or simvastatin stimulates the phosphorylation of ERK1/2 MAPK in mouse periodontal ligament cells. (A) Effects of statins on ERK phosphorylation. The mouse periodontal ligament cells were cultured with 0.1 μM of lovastatin or simvastatin for 5–60 min. After the indicated time, the level of ERK was determined by western blot analysis using the specific antibodies to phospho-ERK1/2. (B) Effects of U0126 on statin-induced ERK phosphorylation. The mouse periodontal ligament cells were exposed to 0.1 μM of lovastatin or simvastatin for up to 60 min after a pretreatment with 10 μM U0126 (MEK1/2 inhibitor) for 2 h. (C) Phosphorylation levels of ERK1/2 were quantified by densitometry and the levels are expressed as the p-ERK/ERK ratio.

induction of bone morphogenetic protein-2. This suggests that the use of statins can be a good strategy for controlling patients with hyperlipidemia and periodontal bone destruction.

Recently, the involvement of the phosphoinositide-3-kinase (PI3K) and MAPK pathways in osteoblast differentiation and skeletal development has been suggested. For example, MAPK stimulation achieved by the selective expression of constitutively active MAPK/ERK1 (MEK-SP) in osteoblasts accelerated the *in vitro* differentiation of calvarial osteoblasts as well as *in vivo* bone development, whereas dominant-negative MEK1 (MEK-DN) was inhibitory (20). In addition, both the basal and the growth factor-stimulated MAPK activity and cell proliferation were inhibited when human osteoblastic cells were transduced with a pseudotyped retrovirus encoding a mutated ERK1 protein with a dominant-negative action against both ERK1 and ERK2 (ERK1DN cells; 21). Expression of the ERK1DN protein suppressed both osteoblast differentiation and matrix mineralization by decreasing the ALP activity and the deposition of bone matrix proteins. Cell adhesion to extracellular matrices was decreased, and cell spreading and migration on these matrices were also inhibited in the ERK1DN cells (21). In a mesenchymal stem cell model of osteoblast commitment and differentiation controlled by bone morphogenetic protein-2, an inhibitor of PI3K or a dominant-negative Akt suppressed osteoblastic differentiation, indicating that the PI3K–Akt signaling cascade is essential for bone morphogenetic protein-2-activated osteoblast differentiation and maturation as well as bone development (22). Lovastatin was also reported to activate PI3K in the plasma membrane, which in turn regulates Akt and ERK1/2 to induce bone morphogenetic protein-2 expression in osteoblast progenitor cells for osteoblast differentiation (23). In human periodontal ligament cells, an ERK1/2 inhibitor suppressed ascorbic acid-induced ALP and osteocalcin gene expression (24).

The present study also examined which signal pathway is involved in statin-induced osteoblast differentiation of mouse periodontal ligament cells. The main focus was on the MAPK/ERK pathway because others reported that the ERK pathway is involved in the differentiation of periodontal ligament cells and osteoblastic cells. Western blot analysis showed that lovastatin and simvastatin enhanced the phosphorylation of ERK within 5 min in mouse periodontal ligament cells. In addition, pretreatment with U0126 inhibited the phosphorylation of ERK in mouse periodontal ligament cells after statin exposure. In the untreated control cultures, ERK phosphorylation was not pronounced, and increased slightly only after 30 min incubation, and the U0126 pretreatment did not cause any significant changes. Mouse periodontal ligament cells treated with 10 μM U0126 almost completely blocked the effects of statin-induced ERK phosphorylation. This means that statin stimulates ERK phosphorylation and might affect the differentiation of mouse periodontal ligament cells. To address whether the ERK pathway affects osteogenic differentiation in mouse periodontal ligament cells, we used ALP to stain the mouse periodontal ligament cells treated with 0.1 μM lovastatin or simvastatin in a differentitation medium with or without 10 μM U0126 (MEK1/2 inhibitor) for 7 d. The ALP staining showed that the intensity of positive staining decreased with U0126 treatment by 40–50% in cells treated with either statin (Fig. 6). These results suggest that lovastatin and simvastatin induce osteoblastic transdifferentiation of

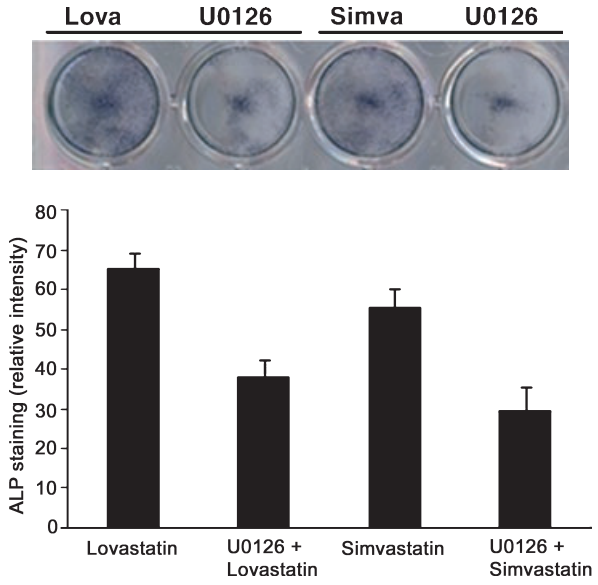

Fig. 6. Effect of the MEK1/2 inhibitor U0126 on statin-induced ALP activity. Cells were induced in a differentiation medium containing 0.1 μM lovastatin or simvastatin, with or without 10 μM U0126 for 7 d, and ALP staining was accomplished and photographed. The intensity of staining was also analysed using image software. The data are expressed as the means ± SD of duplicate samples.

mouse periodontal ligament cells via the ERK1/2 pathway, as reported in osteoblast cells (20,21,23). However, the involvement of PI3K and p38 MAPK in statin-induced osteoblastic differentiation of mouse periodontal ligament cells could not be defined due to the inconsistent results (data not shown).

ERK1/2 are also key mediators of a range of mitogenic signaling pathways, including cytokine- and mechanical stress-induced cell proliferation (25). The present study examined the effects of statins on the cell viability of mouse periodontal ligament cells using an XTT assay. At concentrations of 0.01–0.3 μM, simvastatin and lovastatin did not affect the proliferation of mouse periodontal ligament cells after 24–72 h. However, 1 μM of the statins resulted in some inhibition of cell proliferation. Previous studies reported that statins inhibit cell proliferation of smooth muscle cells (26–28) and endothelial cells (27,29). However, they promote the mitotic activity of fibroblasts (27). In the case of human periodontal ligament cells, 0.1 or 1 μM of simvastatin promoted cell proliferation after 24 h, but 1 μM of simvastatin inhibited proliferation after 72 h (30). These findings indicate that the effect of simvastain and lovastatin on cell proliferation might vary according to the cell type, the type of statin and the concentration.

Periodontal ligament cells and bone marrow mesenchymal stem cells have common biological characteristics (13,14). One study on bone marrow stem cells showed that alendronate, a bisphosphonate used for osteoporosis treatment, stimulates osteogenic differentiation and inhibits adipogenic differentiation in a dose-dependent manner. This effect is mediated by the activation of ERK and JNK(c-Jun N-terminal kinases) (31). Thus, there might be a close relationship between the mevalonate pathway and bone formation in periodontal ligament cells, which requires further study.

Conclusion

The lactone form of statins, lovastatin and simvastatin, at concentrations <1 μM, stimulate the osteoblastic differentiation of mouse periodontal ligament cells via the ERK1/2 pathway. This suggests that these statins can be useful for regenerating periodontal hard tissue by promoting the osteoblastic differentiation of mouse periodontal ligament cells.

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References

1. Sodek J, McKee MD. Molecular and cellular biology of alveolar bone. *Periodontol 2000* 2000;**24:**99–126.
2. Lekic P, McCulloch C. Periodontal ligament cell population: the central role of fibroblasts in creating a unique tissue. *Anat Rec* 2004;**245:**327–341.
3. Melcher AH. On the repair potential of periodontal tissues. *J Periodontol* 1976;**47:**256–260.
4. Zhang D, Yang YQ, Li XT, Fu MK. The expression of osteoprotegerin and the receptor activator of nuclear factor kappa B ligand in human periodontal ligament cells cultured with and without 1alpha, 25-dihydroxyvitamin D3. *Arch Oral Biol* 2004;**49:**71–76.
5. Mundy G, Garrett R, Harris S *et al*. Stimulation of bone formation in vitro and in rodents by statins. *Science* 1999;**286:**1946–1949.
6. Sugiyama M, Kodama T, Konishi K, Abe K, Asami S, Oikawa S. Compactin and simvastatin, but not pravastatin, induce bone morphogenetic protein-2 in human osteosarcoma cells. *Biochem Biophys Res Commun* 2000;**271:**688–692.
7. Ohnaka K, Shimoda S, Nawata H *et al*. Pitavastatin enhanced BMP-2 and osteocalcin expression by inhibition of Rho-associated kinase in human osteoblasts. *Biochem Biophys Res Commun* 2001;**287:**337–342.
8. Parhami F, Mody N, Gharavi N, Ballard AJ, Tintut Y, Demer LL. Role of the cholesterol biosynthetic pathway in osteoblastic differentiation of marrow stromal cells. *J Bone Miner Res* 2002;**17:**1997–2003.
9. Thylin MR, McConnell JC, Schmid MJ *et al*. Effects of simvastatin gels on murine calvarial bone. *J Periodontol* 2002;**73:**1141–1148.
10. Wong RW, Rabie AB. Early healing pattern of statin-induced osteogenesis. *Br J Oral Maxillofac Surg* 2005;**43:**46–50.
11. Sugiyama T, Nakagawa T, Sato C *et al*. Subcutaneous administration of lactone form of simvastatin stimulates ectopic

osteoinduction by rhBMP-2. *Oral Dis* 2007;**13:**228–233.
12. D'Errico JA, Ouyang H, Berry JE *et al*. Immortalized cementoblasts and periodontal ligament cells in culture. *Bone* 1999;**25:**39–47.
13. Pitaru S, Pritzki A, Bar-Kana I, Grosskopf A, Savion N, Narayanan AS. Bone morphogenetic protein 2 induces the expression of cementum attachment protein in human periodontal ligament clones. *Connect Tissue Res* 2002;**43:**257–264.
14. Nakamura T, Yamamoto M, Tamura M, Izumi Y. Effects of growth/differentiation factor-5 on human periodontal ligament cells. *J Periodontal Res* 2003;**38:**597–605.
15. Basdra EK, Komposch G. Osteoblast-like properties of human periodontal ligament cells: an in vitro analysis. *Eur J Orthod* 1997;**19:**615–621.
16. Zhou Y, Hutmacher DW, Sae-Lim V, Zhou Z, Woodruff M, Lim TM. Osteogenic and adipogenic induction potential of human periodontal cells. *J Periodontol* 2008;**79:**525–534.
17. Ruiz-Gaspa S, Nogues X, Enjuanes A *et al*. Simvastatin and atorvastatin enhance gene expression of collagen type 1 and osteocalcin in primary human osteoblasts and MG-63 cultures. *J Cell Biochem* 2007;**101:**1430–1438.
18. Aubin J, Heersche J. Osteoprogenitor cell differentiation to mature bone-forming osteoblasts. *Drug Dev Res* 2000;**49:**206–215.
19. Mundy GR. Statins and their potential for osteoporosis. *Bone* 2001;**29:**495–497.
20. Franceschi RT, Ge C, Xiao G, Roca H, Jiang D. Transcriptional regulation of osteoblasts. *Ann N Y Acad Sci* 2007; **1116:**196–207.
21. Lai CF, Chaudhary L, Fausto A *et al*. Erk is essential for growth, differentiation, integrin expression, and cell function in human osteoblastic cells. *J Biol Chem* 2001;**276:**14443–14450.
22. Mukherjee A, Rotwein P. Akt promotes BMP2-mediated osteoblast differentiation and bone development. *J Cell Sci* 2009; **122:**716–726.
23. Ghosh-Choudhury N, Mandal CC, Choudhury GG. Statin-induced Ras activation integrates the phosphatidylinositol 3-kinase signal to Akt and MAPK for bone morphogenetic protein-2 expression in osteoblast differentiation. *J Biol Chem* 2007;**282:**4983–4993.
24. Mimori K, Komaki M, Iwasaki K, Ishikawa I. Extracellular signal-regulated kinase 1/2 is involved in ascorbic acid-induced osteoblastic differentiation in periodontal ligament cells. *J Periodontol* 2007;**78:**328–334.
25. Chambard JC, Lefloch R, Pouyssegur J, Lenormand P. ERK implication in cell cycle regulation. *Biochim Biophys Acta* 2007;**1773:**1299–1310.
26. van Vliet AK, Negre-Aminou P, van Thiel GC, Bolhuis PA, Cohen LH. Action of lovastatin, simvastatin, and pravastatin on sterol synthesis and their antiproliferative effect in cultured myoblasts from human striated muscle. *Biochem Pharmacol* 1996;**52:**1387–1392.
27. Negre-Aminou P, van Vliet AK, van Erck M, van Thiel GC, van Leeuwen RE, Cohen LH. Inhibition of proliferation of human smooth muscle cells by various HMG-CoA reductase inhibitors; comparison with other human cell types. *Biochim Biophys Acta* 1997;**1345:**259–268.
28. Axel DI, Riessen R, Runge H, Viebahn R, Karsch KR. Effects of cerivastatin on human arterial smooth muscle cell proliferation and migration in transfilter cocultures. *J Cardiovasc Pharmacol* 2000; **35:**619–629.
29. Vincent L, Chen W, Hong L *et al*. Inhibition of endothelial cell migration by cerivastatin, an HMG-CoA reductase inhibitor: contribution to its anti-angiogenic effect. *FEBS Lett* 2001;**495:**159–166.
30. Yazawa H, Zimmermann B, Asami Y, Bernimoulin JP. Simvastatin promotes cell metabolism, proliferation, and osteoblastic differentiation in human periodontal ligament cells. *J Periodontol* 2005;**76:** 295–302.
31. Fu L, Tang T, Miao Y, Zhang S, Qu Z, Dai K. Stimulation of osteogenic differentiation and inhibition of adipogenic differentiation in bone marrow stromal cells by alendronate via ERK and JNK activation. *Bone* 2008;**43:**40–47.