

The Effect of Simvastatin on the Proliferation and Differentiation of Human Bone Marrow Stromal Cells

Statins have been postulated to affect the bone metabolism. Recent experimental and epidemiologic studies have suggested that statins may also have bone protective effects. This study assessed the effects of simvastatin on the proliferation and differentiation of human bone marrow stromal cells (BMSCs) in an ex vivo culture. The bone marrow was obtained from healthy donors. Mononuclear cells were isolated and cultured to osteoblastic lineage. In the primary culture, 10^{-6} M simvastatin diminished the mean size of the colony forming units-fibroblastic (CFU-Fs) and enhanced matrix calcification. At near confluence, the cells were sub-cultured. Thereafter, the alkaline phosphatase (ALP) activities of each group were measured by the time course of the secondary culture. Simvastatin increased the ALP activity in a dose dependent manner, and this stimulatory effect was more evident during the early period of culture. A 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) assay was performed during the secondary culture in order to estimate the effect of simvastatin on the proliferation of human BMSCs. When compared to the control group, simvastatin significantly decreased the proliferation of cells of each culture well. 10^{-6} M of simvastatin also significantly enhanced the osteocalcin mRNA expression level. This study shows that simvastatin has a stimulatory effect on bone formation through osteoblastic differentiation, and has an inhibitory effect on the proliferative potential of human BMSCs

Key Words : Simvastatin; Osteoblasts; Cell Proliferation; Cell Differentiation; Bone Marrow Cells; Stromal Cells

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INTRODUCTION

Mundy et al. first reported evidence showing that statins may have anabolic effects on bone, in which statins stimulated significant new bone formation in rodents (1). Statins act on the mevalonate pathway in osteoblasts and enhance the expression of the bone morphogenic protein-2 (BMP-2), which is an important growth factor for osteoblast differentiation. It is believed that BMP-2 upregulation mediates the bone forming effect of statins. Additionally, statins act on the same pathway upstream of the bisphosphonates in osteoclasts via the inhibition of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, leading to decreased protein prenylation, which is essential for a normal osteoclast function (2).

As a result of these reports, many clinical investigations have reexamined the datasets from observational and cohort trials to assess the effect of statins on the bone metabolism and fracture risk. Some studies have reported beneficial effects

(3-6), whereas others have not (7-9). In a randomized controlled trial, simvastatin had no significant effect on bone mineral density and bone turnover in postmenopausal osteopenic women (10).

In rodents, orally administered statins have been shown to increase the bone volume in some (1, 11), but not all studies (12). Skoglund et al. recently reported that statins enhance the net bone formation and improve the healing of fractures in mice (13). In addition, in vitro studies have shown that statins enhance the osteoblastic synthesis of BMP-2 and promote osteoblastic differentiation in a mouse osteoblastic cell line (14), in a human osteosarcoma cell line (15) and in murine embryonic stem cells (16).

Many of the studies investigating the effects of statins on osteoblasts were performed either on an animal cell line, or another immortalized cell line and studies that examine the influence of statin on the proliferation of bone cells are scarce. In the present study, we investigated whether simvastatin, a

HMG-CoA reductase inhibitor, regulates the proliferation and differentiation of osteoblasts using primary human bone marrow stromal cells.

MATERIALS AND METHODS

Culture of human bone marrow stromal cells

Bone marrow was harvested from the iliac crest of healthy donors. They were donors of bone marrow transplant, and informed consent was obtained from all donors. The mononuclear cells were separated using Ficoll-Hypaque (1.077 g/mL, Sigma-Aldrich Co, St. Louis, MO, U.S.A.) and cultured as described (17). In brief, they were seeded in culture flasks including α -MEM (Sigma) at a density of 4×10^5 cells/mL, and incubated at 37°C in a humidified atmosphere containing 95% air and 5% CO₂. Collagen was not pre-treated in culture flasks. The medium was composed of 20% heat inactivated fetal calf serum (FCS) (Life Technologies, NY, U.S.A.), 100 unit/mL penicillin-streptomycin (Life Technologies), and 10⁻⁸ M vitamin-K (Sigma). On the next day, 10 mM β -glycerophosphate (Sigma) and 50 μ g/mL ascorbic acid were added without changing the media. Subsequently, the culture flasks were left alone without disturbing them so as to promote cell attachment for 4 to 5 days. After the attachment period, the culture medium was replaced with fresh α -minimum essential medium (MEM) including 20% heat inactivated FCS, 100 unit/mL penicillin-streptomycin, 10⁻⁸ M vitamin-K, 10 mM β -glycerophosphate and 50 μ g/mL ascorbic acid. The media was supplemented with simvastatin (dissolved in 75% ethanol, kindly provided by Merck, NJ, U.S.A.) at a concentration of 10⁻⁸ M and 10⁻⁶ M. The cells were then fed at 2-day intervals thereafter. For the subculture, the mononuclear cells were cultured without simvastatin to near-confluence. The cells were then sub-cultured after 0.25% trypsin/1 mM EDTA (Life Technologies) digestion and seeded in 6 well culture plates at a density of 5×10^4 cells/mL. In the secondary culture, the cells were maintained in α -MEM including 10% heat inactivated FCS and treated with either the vehicle or 10⁻⁸ or 10⁻⁶ M simvastatin for the indicated time period.

Staining and determination of colony number and size

Crystal violet staining was performed after 15 days of the primary culture. The cell layers were rinsed with phosphate buffered saline, and stained with 0.5% crystal violet for 5 min at room temperature. The number of colony-forming fibroblastic units (CFU-Fs) was counted in duplicate by both the naked eye and by using inverted optical microscopy (Olympus BH2; Olympus Denmark, Albertslund, Denmark). A single observer who blinded the conditions used in the study counted all the colonies. The CFU-Fs that were 1 mm in diameter or larger were counted. The colony size was determined

by image analysis of the plate using Sigma Scan pro 4.0 for Windows (SPSS, Inc., Chicago, IL, U.S.A.).

Matrix calcification-calcium determination

After 17 days of the primary culture, the cells were washed thoroughly with PBS and incubated overnight at room temperature in 500 μ L of 0.1 N HCl to dissolve the calcium mineral from the calcified matrix. The supernatants were collected and the calcium level was determined using the *o*-cresolphthalein complexone method (Sigma kit, Procedure #587, Sigma). The cells were again washed with PBS and the protein was dissolved in 1 mL of 0.1 N NaOH/0.1% sodium dodecyl sulfate overnight at room temperature. The protein content was determined by the bicinchoninic acid (Sigma) method (18), which was used to normalize the calcium data.

Alkaline phosphatase activity

The alkaline phosphatase (ALP) activities were measured at 3-day intervals for 12 days in the secondary culture period. The HBMS cells in 6-well plates were washed three times with phosphate buffered saline. The cell layers were lysed with 0.1% Triton X-100 at 1 mL per well. 1 M Tris-HCl 500 μ L, 5 mM MgCl₂ 100 μ L and 5 mM *p*-nitrophenyl phosphate (Sigma) 100 μ L were added to the 300 μ L lysate. The samples were incubated for 30 min at 37°C. The absorbance was read immediately after incubation at a wavelength of 410 nm using a plate reader (19). Bovine serum albumin was used as a standard for measuring the protein concentration using the bicinchoninic acid method. The enzyme activities are expressed as U/mg protein.

MTT assay

The 5-diphenyltetrazolium bromide (MTT, thiazoyl blue, Sigma) assay was used to measure the cellular growth and survival (20). The total cell number in each well was estimated from the results of MTT assay. The cells were seeded in 6 well plates and treated in the same manner as with the alkaline phosphatase activity assay. The MTT assay was performed at 5-day intervals for 15 days in the secondary culture period. The MTT was diluted to a final concentration of 0.5 mg/mL in the culture media. The cells were incubated for 2 hr, and the media was carefully removed. The formazan crystals were dissolved in acidified isopropanol (40 mM HCl in isopropanol) and the absorbance was determined at a wavelength of 590 nm.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

The cells were seeded in 6 well culture plates at a density of 1×10^5 cells per well and grown for 12 days. After 2 days of simvastatin treatment, the RNA was extracted using TRIzol®

(Life Technologies) and isolated according to the manufacturer's protocol. A RNA pellet was dissolved in diethylpyrocarbonate treated (DEPC) H₂O, quantified by its absorbance at a wavelength of 260 nm, and stored at -70°C. cDNA was synthesized from 1 µg of the total RNA. The RNA was mixed with a random primer, and incubated for 10 min at 65°C. The 10× RT buffer, MgCl₂ (5 mM), dNTP (1.0 mM of each dATP, dGTP, dCTP, and dTTP), RNase inhibitor (50 U), and AMV-reverse transcriptase (20 U, Roche Molecular Biochemicals, Indianapolis, IN, U.S.A.) were then added, and the reaction was incubated for at least 4 hr at 42°C. The PCR reactions were performed in duplicate. The cDNA (1 µL) was amplified in a 20 µL reaction mixture containing the 10× PCR buffer, MgCl₂ (1.5 mM), dNTPs (0.2 mM of each dATP, dGTP, dCTP and dTTP), primers (0.5 mM for each sense and antisense primer), and *Taq* DNA polymerase (0.025 U/mL, Roche). The sense and antisense primers, 5'-CGGAGTCAACGGATTTGGTCGTAT-3', 5'-AGCCTTCTCCATGGTGGTGAAGAC-3'; 5'-CCTGAAAGCCGATGTGGTC-3', 5'-CTCACA CTCC-TGCCCTAT-3' (Bioneer, Daejeon, Korea), respectively, were used for the amplification of the glyceraldehyde-3-phosphate dehydrogenase (GADH, Bioneer) and osteocalcin. The PCR products were separated in a 3% agarose gel and visualized by ethidium bromide staining. The band intensities were quantitated using Gel Doc 1000[®] and the Molecular Analyst Software Version 1.4.1 (Bio-Rad, U.S.A.). Each cycle consisted of 96°C 45 sec, 62°C 45 sec, 72°C 2 min, for various cycle numbers depending on the gene abundance, terminating in 72°C for 5 min. For semi-quantitative PCR, the linear range of amplification was first established and the PCR cycles were chosen to be within that range. The expression of osteocalcin was compared to GAPDH, which served as the normalizing control.

Statistics

The values are expressed as a mean ± SEM. Statistical analysis

was performed using the Wilcoxon signed rank test. A *p* value <0.05 was considered significant.

RESULTS

Effects of simvastatin on CFU-Fs formation

The mean number of CFU-Fs obtained in the control group and the 10⁻⁸ M simvastatin group was 77 ± 21 and 73 ± 20, respectively. There was a trend for the mean number of CFU-Fs to increase after 10⁻⁶ M treatment (94 ± 21), but this was not statistically significant (Fig. 1, 2A).

The CFU-F sizes were examined to determine if simvastatin influences the proliferative potential of the bone marrow stromal cells. 10⁻⁶ M simvastatin significantly decreased the mean CFU-F size compared to the control (11.6 ± 2.8 mm², 22.9 ± 5.0 mm², *p*<0.05), (Fig. 1, 2B).

Effect of simvastatin on matrix calcification

During the primary culture, the addition of simvastatin stimulated matrix calcification, a late marker of osteoblastic maturation. In the presence of 10⁻⁶ M simvastatin, matrix calcification was increased by 1.6 ± 0.15 fold, compared to the control cultures (*p*<0.05), (Fig. 3).

Alkaline phosphatase activity

The alkaline phosphatase activity reached a maximum value on day 3 of the secondary culture. Simvastatin increased the alkaline phosphatase activity in a dose dependent manner, and the increase in the activity was significant at a statin concentration of 10⁻⁶ M at 3 (*p*<0.01) and 6 days (*p*<0.05) in the culture compared to the control cultures. The stimulating effect of simvastatin was not showed during the late period

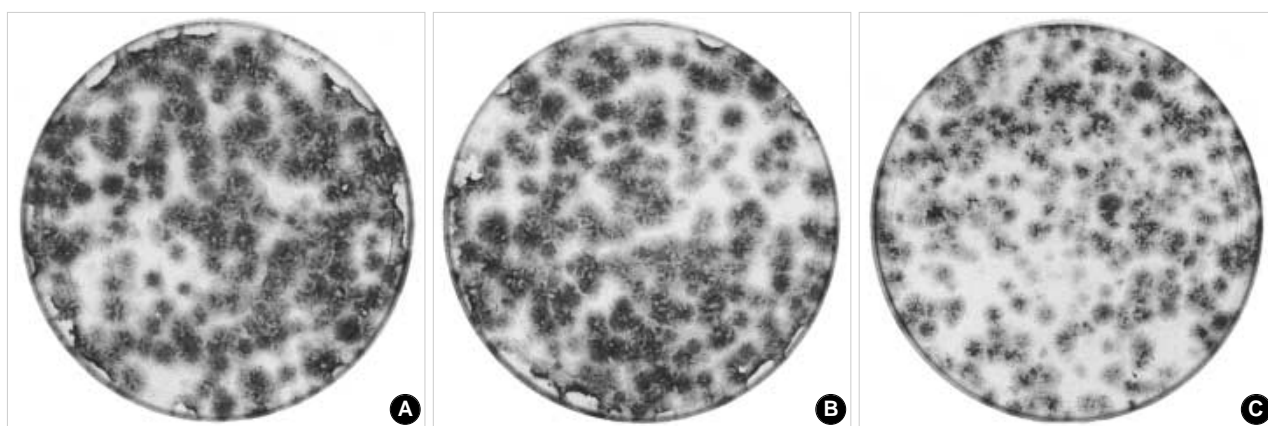


Fig. 1. CFU-F production from human bone marrow cells of the same donor. 4 × 10⁶ nucleated cells, which were derived from the iliac crest, were plated in 10-cm Petri dishes in α -MEM with 20% FCS heat-inactivated, 10 mM β -glycerophosphate and 50 µg/mL ascorbic acid, and grown for 15 days in the absence (A) or presence of 10⁻⁸ M (B) and 10⁻⁶ M (C) simvastatin. Simvastatin was added after the attachment period (4 to 5 days). The colonies were fixed and stained with crystal violet.

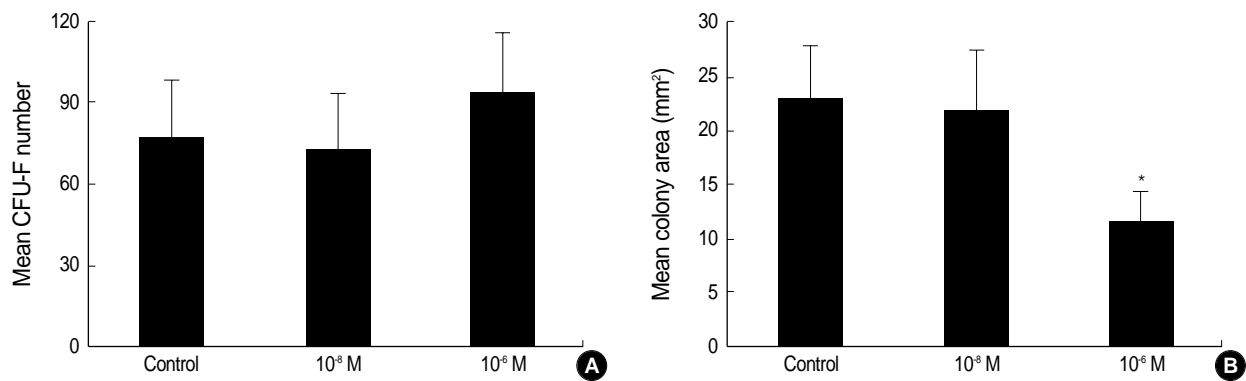


Fig. 2. Effect of simvastatin on the number (A) and the size (B) of CFU-Fs. The bone marrow was aspirated from the iliac crest of 9 young donors. Mononuclear cells were seeded in 10-cm dishes. Simvastatin was added at the appropriate concentrations after the attachment period. After 15 days, the cultures were fixed, stained, and the number and size of the CFU-Fs were determined. The results are shown as a mean \pm SEM. *statistically different from control cultures, $p < 0.05$.

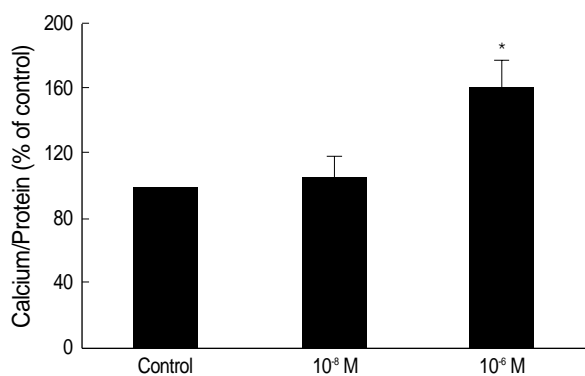


Fig. 3. Effect of simvastatin on matrix calcification. 8×10^5 nucleated cells were plated in 6 well plates in α -MEM with 20% FCS heat-inactivated, 10 mM β -glycerophosphate, and 50 μ g/mL ascorbic acid. After the attachment period, the stromal cells were treated with simvastatin. The calcium content of the matrix was determined as described in the text and normalized to the protein content. The normalized value obtained from the cells incubated under the control conditions was set at 100%, corresponding to 3.26 ± 0.31 μ g calcium/ μ g protein. The results are presented as a mean \pm SEM, $n=11$, each performed in duplicate. *statistically different from control cultures, $p < 0.05$.

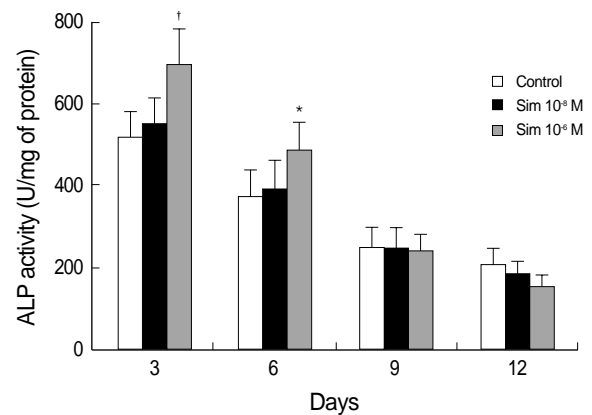


Fig. 4. Effect of simvastatin on the ALP activity in the secondary culture of human marrow stromal cells. After confluence, 1×10^6 stromal cells per well were subcultured in α -MEM with 20% FCS heat-inactivated, 10 mM β -glycerophosphate, 50 μ g/mL ascorbic acid in 6 well plates. The cells were treated with either the vehicle or 10^8 or 10^6 M simvastatin for the indicated time period. The ALP activity was measured as described in the text and normalized to the protein content. The results are shown as a mean \pm SEM. $n=14$, each performed in duplicate. *statistically different from control cultures, $p < 0.05$. †, $p < 0.01$.

of the secondary cultures (Fig. 4).

MTT assay

During the secondary culture, the number of cells progressively increased, and the addition of simvastatin decreased cell proliferation in a dose dependent manner. At 5, 10, and 15 days of culture, 10^6 M simvastatin consistently decreased the number of cells compared to the control ($p < 0.01$) and at 10 and 15 days of culture, 10^8 M simvastatin also decreased the number of cells compared to the control ($p < 0.01$), (Fig. 5).

Osteocalcin mRNA expression

The presence of 10^6 M simvastatin for 48 hr enhanced the

osteocalcin mRNA expression level approximately 1.5 fold compared to that in the unstimulated condition ($p < 0.05$). This effect was not evident after treatment with 10^8 M simvastatin (Fig. 6).

DISCUSSION

This study showed that simvastatin, a HMG-CoA reductase inhibitor, promoted osteoblastic differentiation in the human BMSCs. Simvastatin stimulated the ALP activity, which is an early osteoblastic differentiation marker, and enhanced the expression of osteocalcin, which is a late osteoblastic differentiation marker. Simvastatin also promoted the mineralization of the matrix by the osteoblasts. These results are con-

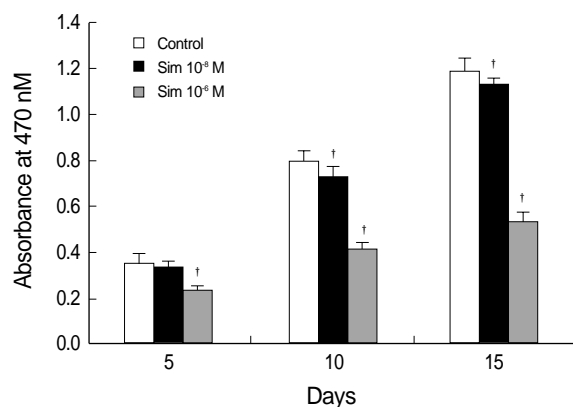


Fig. 5. Effect of simvastatin on cell number in the secondary culture of human marrow stromal cells. The cells were cultured as described in Fig. 4 and treated with the vehicle or either doses (10^{-8} or 10^{-6} M) of simvastatin for the indicated time period. The MTT assay was done as described in the text. The results are presented as a mean \pm SEM, $n=22$, each performed in duplicate. †statistically different from control cultures, $p<0.01$.

sistent with previous observations showing that the statins enhanced osteogenesis in MC3T3-E1 cells (a clonal pre-osteoblastic cell line derived from mouse calvaria) (14) and murine embryonic stem cells (16). Ohnaka *et al.* reported the stimulatory effect of statin on the osteocalcin mRNA expression level in primary culture of human osteoblasts prepared from femur bone fragments (21). All these results and our findings are in good agreement with the concept that statin may differentiate the osteoblasts. This beneficial influence of simvastatin on the bone metabolism might allow the statins to become an effective anabolic agent for treating osteoporosis.

Simvastatin was chosen from various HMG-CoA reductase inhibitors because different statins have been reported to have a varying effect on the bone, with the lovastatin and pravastatin exhibiting the least effect, and statins such as simvastatin, atorvastatin, and cerivastatin exert greater effects (22). In previous experiments investigating the effects of simvastatin on osteoblastic differentiation in MC3T3-E1 cells, significant effects were observed at a concentration of 10^{-7} M and 10^{-8} M simvastatin (14). However, our results showed that the stimulatory effects were evident only at 10^{-6} M simvastatin. It is believed that this discrepancy results from the different culture system and target cells. Human pharmacokinetic studies showed that maximum serum concentration of simvastatin was 1.4×10^{-8} M when 40 mg of simvastatin was administered (23).

Osteoblasts originate from osteoprogenitor cells in the bone marrow stroma termed BMSCs or mesenchymal stem cells (24). During the *in vitro* culture, the BMSCs formed distinct colonies of cells with a fibroblast morphology, colony-forming units-fibroblastic (CFU-Fs) (25). It has been shown that treatment with a number of bone anabolic agents is followed by an increase in the number of CFU-Fs in *ex vivo* cultures (26, 27). In our experiments, the number of CFU-Fs tends

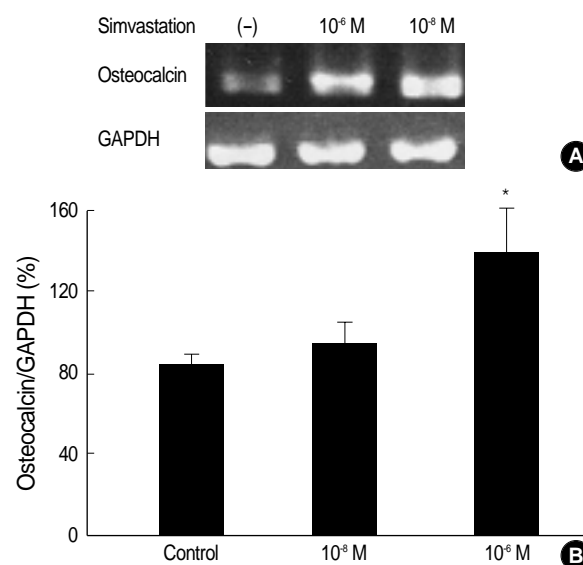


Fig. 6. Effect of simvastatin on osteocalcin gene expression as determined by RT-PCR. The cells were seeded in 6 well culture plates at a density of 1×10^5 cells per well and grown for 12 days. The cells were then treated for 2 days with the vehicle control or either doses (10^{-8} or 10^{-6} M) of simvastatin. 1 μ g of the total RNA from each culture was reverse transcribed and subjected to PCR for osteocalcin analysis. GAPDH mRNA expression was also examined by RT PCR as an internal reference. Representative PCR band (A) and mean data (B) of 22 experiments. The results are presented as a mean \pm SEM, *statistically different from control cultures, $p<0.05$.

to increase after simvastatin treatment, and simvastatin also actively stimulates the osteoblastic differentiation of colonies, as shown by the matrix calcification assay. Therefore, it is likely that statins may stimulate the mobilization of osteoprogenitor cells in the bone marrow, and their presence will also enhance the differentiation of osteoprogenitor cells.

Treatment with BMP-2 increases the ALP activity and osteocalcin production in human BMSCs (28), and stimulates the ALP activity and collagen synthesis in primary cultures of fetal calvarial osteoblasts (29). It is generally agreed that the bone forming effects of the statins are associated with an increased BMP-2 expression level (1). Our results are same as those observed for BMP-2. Although, we did not examine the changes in BMP-2 expression during the simvastatin treatment, it is plausible that BMP-2 exerts its main role also in the osteogenesis of the simvastatin stimulated human BMSCs.

This study also examined whether or not simvastatin affected the proliferation of BMSC in both the primary and secondary cultures. During the primary culture, the addition of 10^{-6} M simvastatin led to a significant decrease in the colony size, which is a marker of the proliferative potential. In the secondary culture, simvastatin significantly decreased the cell number compared to the untreated cells, which was consistent throughout the culture period. This is not due to the cytotoxicity of the dissolving vehicle because equal amounts of ethanol were used in each treatment group and control. Interestingly, these effects are similar to those observed with

BMP-2. Treatment with rhBMP-2 reduced human BMSC growth by 40%, as shown by the DNA content after 2-3 weeks of culture, compared to the untreated cells (30).

To the best of the authors' knowledge, there is no report on the effects of statins on cell growth and proliferation. The implications of these findings are as yet uncertain. Several mechanisms have been suggested to be involved in a bone formation defect: a deficit in the bone marrow stromal cell populations, a decrease in the osteoblastic growth and/or function and a lower proliferation rate of the osteoblast precursors (31). It is difficult to weigh the effects of the proliferation and differentiation on new bone formation; in our opinion, either factors are important. Although the statins have beneficial effects on the differentiation process, if the effects on cell growth and proliferation are considerable, the net effects would be less. The parathyroid hormone, a representative anabolic agent, actually stimulates the proliferation as well as the differentiation of osteoprogenitor cells (32, 33). Besides, the insulin-like growth factor-I (IGF-I), which is another well-known anabolic agent, has been shown to promote osteoblast differentiation and osteoblast proliferation (34, 35). In fact, many studies that examined the effects of statins on the fracture incidence, the bone mineral density, and the biochemical makers of the bone turnover have been published, but the results have been mixed (3-10, 36-42). Additional randomized, prospective, trials with the use of the appropriate statins, doses, and routes would solve these debates. In addition, it is suggested that the inhibitory effects of the statins on bone cell proliferation should be considered before interpreting these results.

In conclusion, this study found that the HMG-CoA reductase inhibitor, simvastatin, is able to increase the alkaline phosphatase activity, the osteocalcin expression level, and the deposition of minerals in human BMSCs. These results suggest that simvastatin has anabolic effects on the bone by promoting osteoblastic differentiation. However, the inhibitory function of the simvastatin on bone cell proliferation also demonstrated that it might interfere with new bone formation. In the process of osteogenesis in vivo, it is difficult to predict whether decreased proliferation or enhanced differentiation would contribute more in the presence of statins, and future studies will be needed to address this issue.

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