

Combined effects of simvastatin and fibroblast growth factor-2 on the proliferation and differentiation of preosteoblasts

JUN-BEOM PARK

Department of Periodontics, Seoul St. Mary's Hospital, College of Medicine,
The Catholic University of Korea, Seoul 137-701, Republic of Korea

Received June 26, 2013; Accepted July 12, 2013

DOI: 10.3892/br.2013.137

Abstract. Simvastatin reportedly promotes osteoblastic and inhibits osteoclastic activity. It increases bone formation when injected subcutaneously over the calvaria in mice. It also increases cancellous bone volume in rats following oral administration. Fibroblast growth factor-2 (FGF-2), a member of the FGF family, is expressed by cells of the osteoblastic lineage. FGF-2 promotes osteoblast proliferation and it is secreted during the healing process of fractures or at bone surgery sites. FGF-2 reportedly regulates bone formation and osteoblast differentiation. In this study, the combined effects of simvastatin and FGF-2 on the proliferation and differentiation of preosteoblasts were investigated and an alkaline phosphatase (ALP) activity test was performed to assess the differentiation. Moreover, the expression of proteins associated with bone formation were measured using western blot analysis. The results demonstrated that the protein content of the cultures grown in osteogenic differentiation media in the presence of FGF-2 at a concentration of 20 ng/ml was higher compared to that of the untreated control cultures. ALP activity was decreased when cells were treated with FGF-2 (2 and 20 ng/ml) and increased when cells were treated with simvastatin. The cultures grown in the presence of 1 μ M of simvastatin and 2 ng/ml of FGF-2 exhibited increased ALP activity when compared to that in the 2 ng/ml FGF-2-only group. The combination of 1.0 μ M simvastatin and 2 ng/ml FGF-2 achieved a higher estrogen receptor- α expression compared to the 2 ng/ml FGF-2-only group. Within the limits of this study, simvastatin enhanced osteoblast differentiation. However, the combined treatment with simvastatin and FGF-2 did not exert synergistic effects on osteoblast differentiation under the current experimental conditions. Future studies are required to evaluate divergent conditions and determine the

selective timing and optimal dosage for the delivery of the agents.

Introduction

Simvastatin reportedly promotes osteoblastic activity and inhibits osteoclastic activity (1). It was shown to increase bone formation when injected subcutaneously over the calvaria in mice and has been demonstrated to increase cancellous bone volume in rats following oral administration (2). The successful use of simvastatin to promote bone formation *in vivo* reportedly depends upon the local concentration. There have been continuous efforts to determine an appropriate treatment protocol (1).

Fibroblast growth factor-2 (FGF-2), a member of the FGF family, is expressed by cells of the osteoblastic lineage. FGF-2 promotes osteoblast proliferation and is secreted during the healing process of fractures or at bone surgery sites (3). It was previously demonstrated that FGF-2 stimulates bone formation and osteoblast differentiation (4). However, the results of a previous study demonstrated that cultures grown in the presence of FGF-2 show an increased value for 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay and a decreased value for alkaline phosphatase (ALP) activity (5). Similarly, FGF-2 was shown to inhibit bone morphogenetic protein-9 (BMP-9)-induced osteogenic differentiation by blocking BMP-9-induced Smads signaling (6).

In this study, the combined effects of simvastatin and FGF-2 on the proliferation and differentiation of preosteoblasts were investigated. The dose-dependent effect of simvastatin and FGF-2 on the proliferation of preosteoblasts was also evaluated. An ALP test was performed to assess differentiation and the expression of proteins associated with bone formation. Specifically, estrogen receptor- α (ER- α) and estrogen receptor- β (ER- β) were measured using western blot analysis to evaluate the underlying mechanism. To the best of the author's knowledge, this study is the first to elucidate the combined effects of simvastatin and FGF-2 on the expression of ER- α in preosteoblasts.

Materials and methods

Cell culture. Mouse calvarial preosteoblasts (MC3T3-E1) were plated and maintained in an α -Minimum Essential

Correspondence to: Dr Jun-Beom Park, Department of Periodontics, Seoul St. Mary's Hospital, College of Medicine, The Catholic University of Korea, 222, Banpo-daero, Seocho-gu, Seoul 137-701, Republic of Korea
E-mail: jbasoonis@yahoo.co.kr

Key words: differentiation, fibroblast growth factor 2, osteoblast, proliferation, simvastatin

Medium (α MEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen), antibiotics (penicillin 100 U/ml and streptomycin 100 μ g/ml; Invitrogen), 10 mM β -glycerophosphate (Sigma, St. Louis, MO, USA) and 50 μ g/ml ascorbic acid (Sigma). The cells were stimulated with simvastatin and FGF-2 at final concentrations of 0.1 μ M (S1) to 1 μ M (S2) for simvastatin and 2 ng/ml (F1) to 20 ng/ml (F2) for FGF-2. The cultures were maintained in a humidified atmosphere with 5% CO₂ and 95% air at 37°C.

Protein measurement. Mouse cells were incubated in α MEM in the presence of ascorbic acid and β -glycerophosphate for 2 days. The protein content was determined based on the Bradford method, using the Coomassie protein assay reagent with a series of bovine serum albumins used as internal standards (7). The absorbance was measured at 595 nm using a microplate spectrophotometer (BioTek, Winooski, VT, USA). The results are presented as the percentage of control values.

ALP activity assay. An ALP assay for osteoblast differentiation was performed after 2 days. The mouse preosteoblasts were lysed with a buffer containing 10 mM Tris-HCl (pH 7.4) and 0.2% Triton X-100. The samples were then sonicated for 20 sec at 4°C and incubated with 10 mM p-nitrophenylphosphate as a substrate in 100 mM glycine buffer (pH 10.5) containing 1 mM MgCl₂ in a water bath at 37°C. The absorbance was measured at 405 nm using a microplate reader (BioTek). In addition, ALP activity was normalized with respect to the total protein content (8,9).

Western blot analysis. The preosteoblasts were washed twice with ice-cold phosphate-buffered saline (PBS) and solubilized with a lysis buffer. The lysates were centrifuged at 16,000 \times g for 20 min at 4°C to remove the nuclear pellet. The supernatants were boiled in a sodium dodecyl sulfate sample buffer containing β -mercaptoethanol. Equal amounts of cell extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride microporous membranes (Immobilon-P; Millipore Corporation, Billerica, MA, USA). The membranes were then blocked for at least 1 h in 0.1% (v/v) PBS and Tween-20 containing 5% (w/v) powdered milk. Each membrane was probed with the desired antibodies, which were diluted in the same buffer at the recommended concentrations. Each membrane was incubated with horseradish peroxidase-conjugated secondary antibody. Subsequently, the washed blot was developed with enhanced chemiluminescence detection kits (5,10). The mouse anti-ER- α and anti-ER- β antibodies and the secondary antibodies conjugated with horseradish peroxidase were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA), Abcam (Cambridge, MA, USA), and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Results

Protein measurement. The protein content in each culture plate was evaluated (Fig. 1). The results demonstrated that the protein content of the cultures grown in the osteogenic differentiation media in the presence of FGF-2 at 20 ng/ml was higher compared to that of the control cultures. However,

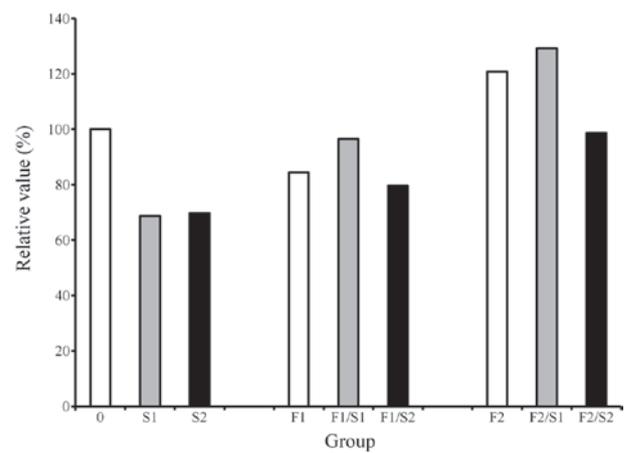


Figure 1. Determination of protein content. S1, 0.1 μ M simvastatin; S2, 1 μ M simvastatin; F1, 2 ng/ml FGF-2; F2, 20 ng/ml FGF-2. FGF, fibroblast growth factor.

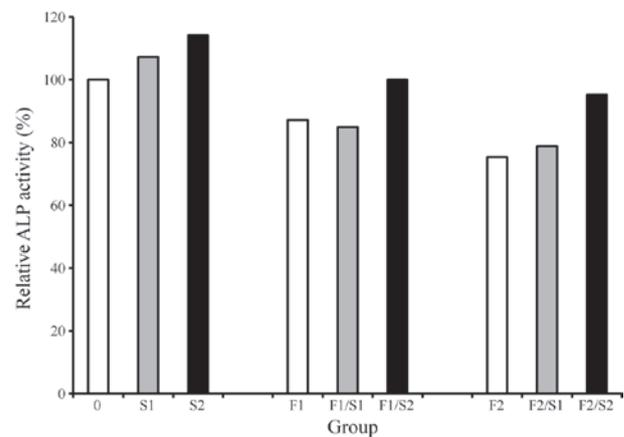


Figure 2. Relative value of alkaline phosphatase (ALP) activity. S1, 0.1 μ M simvastatin; S2, 1 μ M simvastatin; F1, 2 ng/ml FGF-2; F2, 20 ng/ml FGF-2. FGF, fibroblast growth factor.

the addition of 1.0 μ M simvastatin to the cultures uniformly led to a decrease in protein content compared to the simvastatin-unloaded group.

ALP activity assay. ALP activity decreased when cells were treated with FGF-2 (2 and 20 ng/ml) (Fig. 2). The cultures grown in the presence of simvastatin (0.1 and 1.0 μ M) exhibited an increase in ALP activity compared to the control cultures. Similarly, cultures grown in the presence of 1 μ M simvastatin and 2 ng/ml FGF-2 exhibited an increased value of ALP activity when compared to that of the 2 ng/ml FGF-2-only group. The addition of 1 μ M simvastatin to 20 ng/ml of FGF-2 resulted in an increase in ALP activity compared to that of the 20 ng/ml FGF-2 group.

Western blot analysis. A western blot analysis was performed to detect the protein expression following treatment with simvastatin and FGF-2 (Fig. 3). The results demonstrated that the addition of simvastatin (0.1 and 1.0 μ M) appeared to increase the expression of ER- α . Similarly, the combination of 1.0 μ M simvastatin and 2 ng/ml FGF-2 led to a higher ER- α expression compared to that of the 2 ng/ml FGF-2-only group.

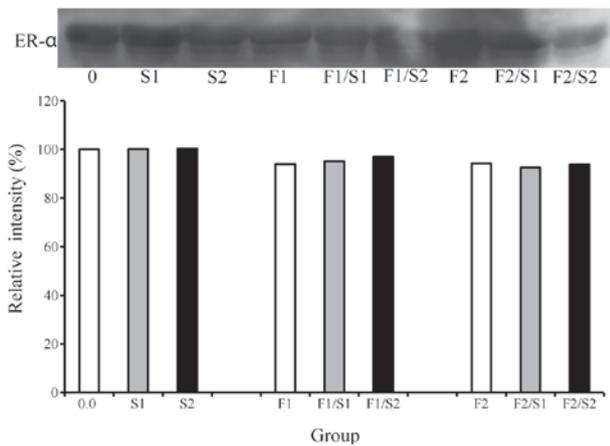


Figure 3. Western blot analysis of the protein expression of estrogen receptor- α . A quantitative analysis of the protein expressions of estrogen receptor- α was conducted. S1, 0.1 μ M simvastatin; S2, 1 μ M simvastatin; F1, 2 ng/ml FGF-2; F2, 20 ng/ml FGF-2. FGF, fibroblast growth factor.

Discussion

In this study, the combined effects of simvastatin and FGF-2 on the protein content, differentiation and protein expression of preosteoblasts under predetermined concentrations (0.1 and 1.0 μ M simvastatin; 2 and 20 ng/ml FGF-2) were investigated. The mechanism of action of simvastatin and FGF-2 on the regulation of protein expression in mouse preosteoblasts was also investigated. In addition, evaluations were performed to determine whether the combination of simvastatin and FGF-2 produced effects additively, synergistically, or competitively.

An increase in protein content was achieved in the 20 ng/ml FGF-2 group under osteogenic differentiation media. This finding was consistent with the results of a previous study demonstrating that FGF-2 affected the proliferation of osteoblasts (5). The treatment of mouse preosteoblasts with simvastatin increased the level of ALP activity. Similarly, previous results demonstrated that cultures grown in the presence of simvastatin exhibited an increased value of ALP activity and mineralization (11). However, treatment with FGF-2 yielded a decreased value of ALP activity. This result was consistent with that of a previous study demonstrating that FGF-2 affected the differentiation of the cells under investigation (5). This study has demonstrated that the addition of 1.0 μ M simvastatin to 20 ng/ml FGF-2 led to an increase in ALP activity compared to that of the 20 ng/ml FGF-2 group. However, the value of ALP activity with the combination of 1.0 μ M simvastatin and 20 ng/ml FGF-2 did not reach the value of the untreated control group.

Western blot analysis was performed to detect the protein expression of ER- α and ER- β to in order to provide information on potential additional mechanisms. The results demonstrated that the addition of simvastatin (0.1 and 1.0 μ M) appeared to increase the expression of ER- α . Similarly, the combination of 1.0 μ M simvastatin and 2 ng/ml FGF-2 led to a higher ER- α expression compared to that of the 2 ng/ml FGF-2-only group.

Estrogens reportedly play a key role in bone formation and bind to estrogen receptors to exert their tissue-specific effects (7,12). The findings of this study suggest that a combination of simvastatin and FGF-2 may partially exert effects

on preosteoblasts through the expression of ER- α (11). The results regarding the effect of the combination of simvastatin and FGF-2 on osteoblastic proliferation and differentiation may be controversial due to the different culture conditions, type of cells, maturation stages of the cells under investigation and differences among species (13,14).

Within the limits of this study, simvastatin enhanced osteoblast differentiation. However, the combined treatment with simvastatin and FGF-2 did not exert synergistic effects on osteoblast differentiation under the current experimental conditions. Therefore, future studies are required to evaluate divergent conditions and determine the selective timing and the optimal dosage for the delivery of these agents.

Acknowledgements

This study was supported by the Seoul St. Mary's Hospital Clinical Medicine Research Program year of 2013 through the Catholic University of Korea.

References

- Park JB: The use of simvastatin in bone regeneration. *Med Oral Patol Oral Cir Bucal* 14: e485-e488, 2009.
- Mundy G, Garrett R, Harris S, *et al*: Stimulation of bone formation in vitro and in rodents by statins. *Science* 286: 1946-1949, 1999.
- Biver E, Soubrier AS, Thouverey C, *et al*: Fibroblast growth factor 2 inhibits up-regulation of bone morphogenic proteins and their receptors during osteoblastic differentiation of human mesenchymal stem cells. *Biochem Biophys Res Commun* 427: 737-742, 2012.
- Zhou L and Ogata Y: Transcriptional regulation of the human bone sialoprotein gene by fibroblast growth factor 2. *J Oral Sci* 55: 63-70, 2013.
- Park JB: Effects of fibroblast growth factor 2 on osteoblastic proliferation and differentiation by regulating bone morphogenetic protein receptor expression. *J Craniofac Surg* 22: 1880-1882, 2011.
- Song T, Wang W, Xu J, *et al*: Fibroblast growth factor 2 inhibits bone morphogenetic protein 9-induced osteogenic differentiation of mesenchymal stem cells by repressing Smads signaling and subsequently reducing Smads dependent up-regulation of ALK1 and ALK2. *Int J Biochem Cell Biol* 45: 1639-1646, 2013.
- Park JB: Low dose of doxycycline promotes early differentiation of preosteoblasts by partially regulating the expression of estrogen receptors. *J Surg Res* 178: 737-742, 2012.
- Park JB: The effects of dexamethasone, ascorbic acid, and β -glycerophosphate on osteoblastic differentiation by regulating estrogen receptor and osteopontin expression. *J Surg Res* 173: 99-104, 2012.
- Park JB: Combination of simvastatin and bone morphogenetic protein-2 enhances differentiation of osteoblastic cells by regulating the expression of phospho-Smad1/5/8. *Exp Ther Med* 4: 303-306, 2012.
- Park JB: Effects of doxycycline, minocycline, and tetracycline on cell proliferation, differentiation, and protein expression in osteoprecursor cells. *J Craniofac Surg* 22: 1839-1842, 2011.
- Park JB, Zhang H, Lin CY, *et al*: Simvastatin maintains osteoblastic viability while promoting differentiation by partially regulating the expressions of estrogen receptors α . *J Surg Res* 174: 278-283, 2012.
- Pinzone JJ, Stevenson H, Strobl JS and Berg PE: Molecular and cellular determinants of estrogen receptor α expression. *Mol Cell Biol* 24: 4605-4612, 2004.
- Kim CH, Cheng SL and Kim GS: Effects of dexamethasone on proliferation, activity, and cytokine secretion of normal human bone marrow stromal cells: possible mechanisms of glucocorticoid-induced bone loss. *J Endocrinol* 162: 371-379, 1999.
- Ishida Y and Heersche JN: Glucocorticoid-induced osteoporosis: both in vivo and in vitro concentrations of glucocorticoids higher than physiological levels attenuate osteoblast differentiation. *J Bone Miner Res* 13: 1822-1826, 1998.