Simvastatin-loaded porous implant surfaces stimulate preosteoblasts differentiation: an in vitro study

Fan Yang, PhD, DDS, Shi-fang Zhao, MD, DDS, Feng Zhang, PhD, DDS, Fu-ming He, PhD, DDS, and Guo-li Yang, PhD, DDS
Hangzhou, China
DEPARTMENT OF ORAL IMPLANTOLOGY, STOMATOLOGY HOSPITAL, MEDICAL SCHOOL, ZHEJIANG UNIVERSITY

Objective. Recent studies demonstrate that simvastatin stimulates bone formation, suggesting the potential application in dental implantology. In this study, our lab developed a simvastatin-loaded titanium porous surface. The aim was to investigate the effect of simvastatin-loaded titanium surfaces on the promotion of osteogenesis in preosteoblasts (MC3T3-E1) in vitro.

Study design. The control group consisted of cells cultured on titanium disks without any intervention for different time intervals (4, 7, and 14 days), and the experimental groups (simvastatin-loaded groups) consisted of cells cultured on titanium disks that were preincubated in varying concentration (10^{-7} mol/L, 10^{-6} mol/L, 10^{-5} mol/L, and 10^{-4} mol/L) of simvastatin for the same time intervals of the control group. Alkaline phosphatase (ALP) activity, type I collagen synthesis, and osteocalcin release were used to measure the cellular osteoblastic activities.

Results. All simvastatin-loaded groups showed increased ALP activity compared with the control group at every time point, especially the 10^{-7} mol/L group, which significantly increased the activity almost fourfold at 4 days (P < .05). In the type I collagen synthesis assay, all simvastatin-loaded groups showed an increase, and the effect was inverse dose dependent (maximal at 10^{-7} mol/L). This stimulatory effect of simvastatin was also observed in the osteocalcin release assay (P < .05; at 10^{-7} mol/L, 10^{-6} mol/L, maximal at 10^{-7} mol/L).

Conclusion. These results indicate that simvastatin-loaded porous implant surfaces promote accelerated osteogenic differentiation of preosteoblasts, which have the potential to improve the nature of osseointegration. (Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2011;111:551-556)

The endosseous dental implant has become a scientifically accepted and well documented treatment for fully and partially edentulous patients.1-4 Titanium and its alloys are the materials of choice clinically, because of their excellent biocompatibility and superior mechanical properties. However, these materials are generally considered to be bio-inert, and conventional dental implant surgery recommends that it takes up to 3 months on average to achieve osseointegration. Several attempts have been made to improve and accelerate osteointegration by modification of surface properties, such as introducing bioactive factors to titanium surfaces. Of these, some osteogenic drugs have been applied to implant surfaces. For example, bisphosphate-loaded implant surfaces have been reported to improve implant osseointegration. However, bisphosphates are antiresorptive agents that have beneficial effects for the patients on preventing further bone loss, and the effect on increasing the bone mass is modest.5-7

Statins are commonly prescribed drugs that inhibit 3-hydroxy-3-methylglutaryl coenzyme reductase to decrease cholesterol biosynthesis by the liver, thereby reducing serum cholesterol concentrations and lowering the risk of heart attack.8 Recently, it was reported that a kind of liposoluble statin, simvastatin, could induce the expression of bone morphogenetic protein (BMP) 2 mRNA and that, as a result, it promoted bone formation on the calvaria of mice following daily subcutaneous injections.9 Subsequent evidence for the metabolic effect of statins on bone in vivo has accumulated.10 Simvastatin given perorally to adult rats increased cancellous bone mass and increased cancellous bone compressive strength.11 Furthermore, Ayukawa et al.12 confirmed that topical application of statins to alveolar bones increased bone formation and concurrently suppressed osteoclast activity at the bone-healing site. In addition, several clinical studies reported that statin use is associated with increased bone mineral density.13,14

The potential use of simvastatin as stimulators of bone anabolism suggests that they may have widespread application in the dental implantology field. To date, few studies have reported the simvastatin use

Department of Oral Implantology, Stomatology Hospital, Medical School, Zhejiang University, Hangzhou, China. Received for publication Feb 11, 2010; returned for revision Jun 7, 2010; accepted for publication Jun 25, 2010.
1079-2104/5 - see front matter
© 2011 Mosby, Inc. All rights reserved.
doi:10.1016/j.tripleo.2010.06.018
related dental implants, and most were limited to systemic administration. For example, Du et al. investigated the effect of simvastatin by oral administration on implant osseointegration in osteoporotic rats and found that it significantly improved bone integration with the implant. Another animal study showed that the intraperitoneal administration of simvastatin increased implant-bone contact ratio and bone density and implied that simvastatin might have the potential to improve the nature of osseointegration.

Knowing the benefits of achieving successful implant osseointegration in the patients that have compromised bone healing, or want early implant loading, we designed a simvastatin-loaded porous titanium implant surface, and the purpose of this study was to investigate cell responses to the drug-loaded surface.

MATERIALS AND METHODS

Surface treatment of titanium disk

Flat pure titanium (grade III) disks (25 mm diameter × 1.5 mm thickness) were treated according to our previously reported procedures. In brief, samples were polished, sandblasted with green silicon carbide at the pressure of 4 MPa, and washed for 15 minutes in an ultrasonic cleaner with acetone, 75% alcohol, and then distilled water. Subsequently, the specimens were chemically treated with a solution containing 0.11 mol/L HF and 0.08 mol/L HNO₃ at room temperature for 10 minutes and dried in an oven at 50°C for 24 hours. Then, the specimens were immersed into a solution containing 5.80 mol/L HCl and 8.96 mol/L H₂SO₄ at 80°C for 30 minutes and dried in an oven at 50°C for 24 hours.

Preparation of simvastatin coating on porous surfaces

Simvastatin was dissolved in 75% ethanol and prepared at concentrations that ranged from 10⁻⁷ mol/L to 10⁻¹ mol/L. Before the preparation of simvastatin, all of the disks were ultrasonically cleaned in acetone, 100% alcohol, and distilled water for 15 minutes each. Then the disks were immersed into different simvastatin solutions for 48 hours. Control group disks were immersed in 75% ethanol alone for 48 hours. All disks were sterilized with ultraviolet light for 1 hour before cell culture. Surface topography of titanium disks were determined by field scanning electron microscopy (FSEM; FEI, Sirion 100, The Netherlands).

Cell culture and seeding

Mouse preosteoblast cells (MC3T3-E1) were cultured in alpha–Minimum Essential Medium (Gibco, Tulsa, OK, USA) supplemented with 10% fetal bovine serum (Gibco) and maintained at 37°C in a 5% CO₂ humidified atmosphere (standard culture conditions). When cells were seeded at a density of 1 × 10⁵ on each disc, basic medium was supplemented with 10 mmol/L β-glycerolphosphate (Sigma-Aldrich, St. Louis, MO, USA) and 50 mg/L ascorbin-2-phosphate (Sigma-Aldrich). Medium was changed every 3 days.

Alkaline phosphatase activity assay

The ALP activity was determined using a commercial phosphatase substrate kit (Wako, Japan), which is a colorimetric end point assay measuring the enzymatic conversion of p-nitrophenyl phosphate to the yellowish product p-nitrophenol in the presence of ALP. After gentle removal of culture medium and washing with PBS, the cells were lysed by CellLytic Buffer (Sigma). A 20-μL volume of cell lysate mixed with 100 μL working assay solution was shaken for 1 minute with a plate mixer and then incubated at 37°C for 15 minutes. Then the addition of 80 μL stop solution to each well terminated the reaction. After that, the 96-well plate was shaken for another 1 minute and read at 405 nm with a spectrophotometer. ALP activity was calibrated by per-unit total cellular protein. Total protein was determined with a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). ALP activity was expressed as nanomole of p-nitrophenol liberated per microgram of total cellular protein per hour.

Type I collagen synthesis assay

The synthesis of collagen type I was measured as the release of extracellular matrix protein into the culture medium by mouse collagen type I alpha-1 enzyme-linked immunosorbent assay kit (Uscn Life Science, Wuhan, China). Briefly, we incubated 100 μL cell culture medium sample and standards at 37°C for 2 hours and then removed them. Then, we added 100 μL Detection Reagent A working solution to each well and incubated for 1 hour at 37°C. After washing the wells with wash buffer, 100 μL Detection Reagent B working solution was added and incubated for 30 minutes at 37°C. Then we added 90 μL of substrate solution to each well and incubated for 15–25 minutes at 37°C in dark. After adding 50 μL of stop solution, absorbance was measured at 450 nm on a spectrophotometer. Data were expressed as nanograms of mouse type I collagen in the medium.

Osteocalcin release assay

The production of osteocalcin was measured as the release of extracellular matrix protein into the culture medium by Mouse Osteocalcin EIA Kit (Biomedical Technologies, Stoughton, MA, USA). Briefly, a 25 μL cell culture medium sample and 100μL osteocalcin antisera were placed in a 96-well EIA plate and
incubated at 4°C for 24 hours. The well was washed with wash buffer, added 100 μL Streptavidin-horseradish reagent, and then incubated at room temperature for 30 minutes. Then 50 μL TMB conjugated hydrogen peroxide solution were added to each well and allow incubating at room temperature for 15 minutes. When adding 100 μL acidic stop solution, absorbance was measured at 450 nm on a spectrophotometer. Data were expressed as ng mouse osteocalcin in the medium per milliliter medium.

Statistical analysis
All the cell and biological analysis results were expressed as mean and standard deviation (SD). At each investigation point, one-way ANOVA test was carried out to determine the statistical significance between control group and simvastatin-loaded groups. \( P \leq 0.05 \) were considered to indicate statistical significance. All statistical tests were carried out using SPSS (version 12.0; SPSS, Chicago, IL, USA).

RESULTS

FSEM observation
Figure 1 shows the FSEM pictures of plates without or with the treatment of simvastatin solution \( (10^{-7} \text{ mol/L}) \). Microscopic evaluation demonstrated that the control surfaces were quite irregular, multilevel pores appeared on the surfaces, and treatment with simvastatin solutions did not destroy the roughened surfaces.

ALP activity
On average, simvastatin-loaded titanium had an increased effect on MC3T3-E1 ALP activities at different concentrations and at every time point investigated (Fig. 2), especially at an early stage (4 days). At 4 days, the \( 10^{-7} \) mol/L group significantly increased activity almost fourfold \( (P < 0.05) \). At 7 days, the ALP level of the \( 10^{-7} \) mol/L, \( 10^{-6} \) mol/L, \( 10^{-5} \) mol/L, and \( 10^{-4} \) mol/L groups increased, although only \( 10^{-7} \) mol/L and \( 10^{-6} \) mol/L produced significant increases: by 23% and 17%, respectively \( (P < .05) \). At 14 days, significant increases of 100% in the \( 10^{-7} \) mol/L group \( (P < .05) \).

Type I collagen synthesis
Figure 3 shows type I collagen synthesis level of MC3T3-E1 cells cultured in control and simvastatin groups. A time-dependent stimulatory effect in the simvastatin-loaded groups on type I collagen synthesis was observed. And at the same time point, the results demonstrated reverse dose-dependent relation within \( 10^{-7} \) mol/L to \( 10^{-4} \) mol/L. At 4 days, the \( 10^{-7} \) mol/L, \( 10^{-6} \) mol/L, and \( 10^{-5} \) mol/L groups showed significant increases compared with the control group \( (P < .05) \), and the increase ratios were 177%, 123%, and 100%, respectively. When cultured for 7 days, significant increases were found in the \( 10^{-7} \) mol/L and \( 10^{-6} \) mol/L groups, and the rates of increase were 58% and 42%.
respectively ($P < .05$). At 14 days, no significant difference was observed.

**Osteocalcin release**

The osteocalcin production released into cell culture medium at day 14 is shown in Fig. 4. It demonstrated that incubation with $10^{-7}$ and $10^{-6}$ mol/L simvastatin enhanced the expression of osteocalcin to a statistically significant extent ($P < .05$) compared with the unstimulated conditions in MC3T3-E1 cells, and this increase was dose-dependent. The rates of increase were 70% and 61% for $10^{-7}$ to $10^{-6}$ mol/L, respectively.

**DISCUSSION**

In this study, we demonstrated that simvastatin-loaded porous titanium surface potently increased ALP activity and extracellular accumulation of proteins such as osteocalcin and type I collagen in mouse preosteoblast MC3T3-E1 cells. In addition, the results showed that a relatively low concentration of simvastatin ($10^{-7}$ mol/L) might be the optimal concentration for the differentiation of osteoblasts.

Our findings were similar to some earlier reports on the osteogenic effects of statins. Mundy et al. first reported the bone anabolic effect of simvastatin both in vivo and in vitro. Subsequent in vitro studies described the stimulation of osteogenic differentiation and concurrent inhibition of adipogenic differentiation of bone marrow mesenchymal cells by simvastatin. Furthermore, studies confirmed that simvastatin could potently induce mRNAs encoding osteoblast differentiation markers such as BMP-2, ALP, type I collagen, bone sialoprotein, and osteocalcin. Ayukawa et al. found that the administration of simvastatin increased the value of both bone contact ratio to the implant and bone density and suggested that this drug might have the potential to improve the nature of osseointegration.

The intimate action mechanisms of the stimulatory effect of simvastatin on bone formation have not been fully defined. BMP-2-mediated action was most accepted, as first described by Mundy et al. Maeda et al. demonstrated that simvastatin initially enhanced BMP-2 expression and then induced synthesis of differentiation markers characteristic of late osteoblast stages, and suppressed gene expression of matrix metallopreinase 13 (collagenase-3) simultaneously. Nitric oxide production through statin-induced up-regulated endothelial nitric oxide synthase also played some key roles in regulating osteoblast differentiation and bone formation. And calcyclin (S100A6), a Ca$^{2+}$ ion-binding protein, was identified by proteomic analysis to be significantly up-regulated when treated with simvastatin and suggested to play an important role in an anabolic effect of simvastatin on bone. Further studies are needed to clarify the mechanisms.
The route of simvastatin administration should be pointed out in this study. Systemic administration has been shown to facilitate osteogenesis process.\textsuperscript{15} However; systemic administration required much higher doses, because they are metabolized in the liver which may cause an insufficient concentration for stimulating topical bone formation. In this context, topical administration might be a superior way to attain local bone augmentation. But conventional local injection also has some limitations and has failed to sustain the simvastatin in the desired area. The results of the present study highlighted the feasibility of administration of simvastatin by adsorption from the implant surface. This was of great convenience for clinical practice.

It is a simple treatment of immersing implants into simvastatin-dissolved ethanol solution before sterilization. FSEM observations demonstrated that simvastatin-dissolved ethanol solution did not destroy the roughened surfaces. The advantage of this method is to preserve the topography of the implant when introducing bone-forming drug (factor) onto the surface. Thus, accelerating early-stage bone formation can be achieved owing to the anabolic effect of simvastatin, which is especially beneficial to patients who have compromised bone healing or want early implant loading. It is noted that the concentration of the drug and the treatment time span are the 2 important parameters that should be taken into account. However, the precise quantity of simvastatin could not be determined, because the effective concentration was too low. Further studies are needed to investigate the effects of simvastatin-loaded titanium implants on osseointegration in animal experiments.

In conclusion, These results indicate that simvastatin-loaded porous implant surfaces promote accelerated osteogenic differentiation of preosteoblasts and have the potential to improve the nature of osseointegration.

The authors thank Zhejiang Guangci Medical Appliance Company for delivering the experimental implants and disks. They also gratefully acknowledge the financial support from Zhejiang Provincial Medical and Health Specialized Research Fund for Excellent Youth Talents.

REFERENCES

21. Maeda T, Matsunuma A, Kurahashi I, Yanagawa T, Yoshida H,


Reprint requests:
Guo-li Yang
Department of Oral Implantology
Stomatology Hospital
Medical School
Zhejiang University
Yan’an Road
Hangzhou
China
yangguoli123456@yahoo.com.cn