Journal of
PERIODONTAL RESEARCH

J Periodont Res 2011; 46: 483–490

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2011.01364.x

Statins regulate interleukin-1β-induced RANKL and osteoprotegerin production by human gingival fibroblasts

S. H. Stein[1], I. N. Dean[1,*], S. Y. Rawal[1], D. A. Tipton[2]

[1]Department of Periodontology, College of Dentistry, University of Tennessee Health Science Center, Memphis, TN, USA and [2]Department of Bioscience Research, College of Dentistry, University of Tennessee Health Science Center, Memphis, TN, USA

Stein SH, Dean IN, Rawal SY, Tipton DA. Statins regulate interleukin-1β-induced RANKL and osteoprotegerin production by human gingival fibroblasts. J Periodont Res 2011; 46: 483–490. © 2011 John Wiley & Sons A/S

Background and Objective: Three-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase competitive inhibitors, or 'statins', are widely used for lowering cholesterol and thereby reducing the risk of a heart attack. Recent data suggest that statins influence metabolic bone activity by their actions on three molecules: RANKL; RANK; and osteoprotegerin (OPG), the soluble decoy receptor for RANKL. The purpose of this study was to evaluate OPG and RANKL production in resting and interleukin-1β (IL-1β)-activated human gingival fibroblasts (HGFs), and to determine the effect of statins on their production.

Material and Methods: Fibroblasts were pre-incubated with atorvastatin or simvastatin for 24 h in serum-free medium, and then incubated with IL-1β for 6 d. The concentration of OPG or RANKL in culture supernatants was measured by specific ELISA. Data were analyzed using analysis of variance and Scheffe's F procedure for *post hoc* comparison.

Results: IL-1β (1×10^{-8} M) stimulated a significant increase in the production of OPG on days 1, 3 and 6. There was a trend towards an increase in RANKL production as a result of stimulation with IL-1β. Both statins, at multiple concentrations, significantly increased the constitutive RANKL/OPG ratio. Only atorvastatin at the highest concentration (5×10^{-6} M) significantly increased the IL-1β-stimulated RANKL/OPG ratio.

Conclusion: IL-1β significantly increased OPG production by HGFs. The statins differed minimally in their effects on OPG and RANKL production by resting and IL-1β-activated HGFs. Both statins increased constitutive RANKL/OPG ratios, but generally not IL-1β-stimulated ratios. Thus, statins may influence the production of RANKL and OPG by HGFs to favor bone catabolism, under noninflammatory conditions.

Dr Sidney H. Stein, Department of Periodontology, UTHSC-College of Dentistry, 875 Union Avenue, Memphis, TN 38163, USA
Tel: 901 448 6242
Fax: 901 448 6751
e-mail: sstein@uthsc.edu
*Currently in private practice in Hamden, CT, USA.

Key words: fibroblasts; osteoprotegerin; RANKL; statins

Accepted for publication February 7, 2011

Three-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) competitive inhibitors, also known as statins, are widely used for lowering the levels of low-density lipoprotein cholesterol and reducing the risk of major coronary events (1). Statins differ in their mode of derivation (as either synthetic or fermentation products), in their lipophilicity

and in potency. Examples of fermentation-derived statins are simvastatin, pravastatin and lovastatin, while atorvastatin, cerivastatin, fluvastatin, pitavastatin and rosuvastatin are synthetic statins. Synthetic statins have different chemical groups attached to the decalin-ring structure, which affords them tighter bonding to the HMG receptor and higher potency (2). Statins also exhibit anti-inflammatory properties independent of their effects upon cholesterol. Reports have shown that their ability to lower the systemic levels of C-reactive protein may provide additional cardioprotection (3,4).

Recent data suggest that statins influence bone metabolic activity by stimulating new bone formation both *in vivo* and *in vitro*. Mundy *et al*. (5) identified statins as a potent activator of bone morphogenetic protein-2 (BMP-2), an important stimulator of osteoblastic differentiation. *In vitro* studies have shown that simvastatin, mevastatin and atorvastatin stimulated *BMP2* transcription and also increased, by twofold, the endogenous expression of *BMP2* mRNA and BMP-2 protein in human MG63 osteoblastic cells (6). Yazawa *et al*. (7) reported a dose-dependent stimulation of alkaline phosphatase activity in human periodontal ligament (PDL) cells by simvastatin, with the peak effect noted at 1 μM. Statins also stimulated the production of osteoprotegerin (OPG), which may contribute to their bone-sparing effect (8). This effect was amplified by the blockage of mevalonate-derived intermediates, including farnesyl-pyrophosphate and geranyl-pyrophosphate, two agents necessary for the activation of osteoclasts. Thus, statins may decrease bone resorption by inhibiting the HMG-CoA reductase pathway (9).

These findings suggest that statins could be considered as potential agents for treating osteoporosis and other diseases of bone loss, such as periodontal disease (10,11). Both diseases share common pathways for bone resorption and increased levels of inflammatory mediators such as interleukin (IL)-1β, tumor necrosis factor-α and IL-6. IL-1β is a potent multifunctional cytokine that activates a wide range of factors, including MMPs, nitric oxide synthase, prostanoids and other cytokines (12). IL-1β has been established as a primary factor associated with the pathogenesis of periodontal disease (13–15).

Bone apposition and bone resorption associated with periodontitis are governed by the interaction of cytokines in the inflamed tissue, and are produced by immune cells such as B- and T lymphocytes, and also by resident cells such as human gingival fibroblasts (HGF) and periodontal ligament (PDL) cells. Additionally, three molecules, members of the tumor necrosis factor ligand and tumor necrosis factor receptor superfamilies, regulate the process of osteoclast formation via cell-to-cell interactions (16). The first molecule, RANKL, is expressed on hematopoietic stromal cells and periosteal osteoblasts, as well as on fibroblasts and on T- and B lymphocytes. RANKL is expressed on osteoblasts as a membrane-bound protein or cleaved into soluble form. RANKL interacts with its corresponding receptor, RANK, on mononucleated osteoclast precursors and induces their activation to multinuclear bone-resorbing osteoclasts (17). The effects of RANKL are blocked by its soluble decoy receptor, OPG, thus inhibiting osteoclast differentiation, activation and survival (18). When the concentrations of OPG are high relative to RANKL expression, OPG binds RANKL, inhibiting the RANK–RANKL interaction, which leads to reduced formation of osteoclasts and apoptosis of pre-existing osteoclasts (19). This environment favors bone formation. In the opposite scenario, when the levels of OPG are low relative to the levels of RANKL, RANKL is available to bind RANK on osteoclast precursors and bone resorption is favored. Bostanci *et al*. (20) reported elevated gingival crevicular fluid levels of RANKL/OPG in periodontitis patients compared with healthy controls. Jin *et al*. (21) utilized a rat model and a human OPG–Fc fusion protein to support the hypothesis that RANKL inhibition by OPG may provide an important therapeutic strategy for the prevention of progressive alveolar bone loss.

RANKL and OPG are produced by many cells, including HGF and PDL cells (22). Some studies report an increase in OPG production by HGF when stimulated by lipopolysaccharide (LPS) from *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* (23,24). Another study reported elevated expression of *RANKL* mRNA and reduced expression of *OPG* mRNA by HGF, resulting in an increased RANKL/OPG expression ratio induced by *P. gingivalis*. (25). Therefore, HGF may play a significant role in the RANK/RANKL/OPG balance at the connective tissue/bone interface in health and in disease. While a limited number of reports have focused on OPG/RANKL production by HGF following stimulation with LPS, no studies have systematically evaluated the effect of IL-1β or statins on the production of both OPG and RANKL by HGF. Therefore, the purpose of this study was to evaluate OPG and RANKL production in resting and IL-1β-activated HGF in the presence and absence of a fermentation-derived and a synthetically derived statin.

Material and methods

Human gingival fibroblasts

Normal HGF were used in this study and were established from explants of noninflamed gingival tissue from healthy individuals using standard techniques (26). The fibroblasts were routinely cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM; Invitrogen Corp., Carlsbad, CA, USA) supplemented with 10% (v/v) newborn calf serum (Invitrogen) and 100 μg/mL of gentamicin (Sigma-Aldrich Corp., St Louis, MO, USA) (complete medium). Cells between passages seven and 15 were used in the experiments (27).

Statins and IL-1β

Atorvastatin was provided by Pfizer (Groton, CT, USA) at no cost through a material transfer agreement.

Simvastatin was purchased from Sigma-Aldrich. Both drugs are soluble in dimethylsulfoxide (DMSO; Sigma-Aldrich). Simvastatin is a synthetic derivative of a fermentation product of *Aspergillus terreus*. It is the only statin available in generic form. Atorvastatin is a completely synthetic product and has been found to be more potent than other statins at the same concentration and same incubation time. Human recombinant IL-1β was from R&D Systems (Minneapolis, MN, USA).

Determination of cytotoxic effects of statins on HGF

Cytotoxicity of the statins was assessed by determining their effects on the ability of the HGF to cleave the tetrazolium salt (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) (MTT) to a formazan dye, using a kit from Boehringer Mannheim Corp. (Indianapolis, IN, USA). Individual wells of 96-well microtiter tissue culture plates (Becton Dickinson Labware, Franklin Lakes, NJ, USA) were seeded with 2.5×10^4 HGF in 0.2 mL of complete medium. The HGF were incubated overnight at 37°C. The medium was then removed and the HGF were washed with phosphate-buffered saline then resuspended in serum-free medium supplemented with 100 μg/mL of gentamicin (DMEM-gent), ± simvastastin or atorvastatin (0.01 nM–5 μM) (final DMSO concentration = 0.1%). The HGF were exposed to statins for 7 d (the period of time the HGF were exposed to the statins in the experiments described below for detecting OPG and RANKL) before the addition of MTT (0.5 mg/mL). The HGF were then incubated for 4 h at 37°C, and purple formazan crystals produced from the MTT by metabolically active HGF were solubilized by overnight exposure at 37°C to a solubilization solution provided in the kit. The absorbance was read at 595 nm using a microtiter plate spectrophotometer. The results of five experiments, each with triplicate samples, were expressed as a percentage relative to the absorbance of the control (i.e. $A_{595\text{nm}}$ in HGF exposed to DMEM-gent only).

Determination of constitutive and IL-1β-stimulated production of RANKL and OPG

Individual wells of six-well culture plates (Corning Inc., Corning, NY, USA) were seeded with 3×10^5 HGF in complete medium and cultured overnight at 37°C. The medium was removed, the HGF were washed with phosphate-buffered saline, and then DMEM-gent, with or without IL-1β (0.01–10 nM), was added. Aliquots of 0.5 mL were collected on days 1, 3 and 6, and stored frozen at −80°C until assayed. Production of OPG and RANKL was measured in the HGF supernatants using the Human DuoSet OPG ELISA Development System (R&D Systems) and a RANKL ELISA (Pepro Tech, Inc., Rocky Hill, NJ, USA), respectively, following the manufacturers' instructions. Absorbance values were read using a microtiter plate spectrophotometer, and the results of two experiments, each with triplicate samples, were expressed as ng/mL (OPG) or pg/mL (RANKL), then converted to a percentage relative to the control value (i.e. the amount of OPG or RANKL produced by cells exposed to DMEM-gent only, set at 100%).

Determination of effects of statins on constitutive or IL-1β-stimulated production of RANKL and OPG

These experiments were essentially carried out as described above, except that in some cases the HGF were pre-incubated for 24 h with atorvastatin or simvastatin (0.01 nM–5 μM) before the addition of IL-1β (0.1 nM). In these experiments, the media were harvested on day 6 only. Samples were assayed using ELISA to measure the levels of OPG or RANKL. Absorbance values were read using a microtiter plate spectrophotometer, and the results of three experiments, each with triplicate samples, were expressed as the amount of OPG or RANKL (ng/mL or pg/mL, respectively). These results were converted to a percentage relative to the control value (i.e. the amount of OPG or RANKL produced by cells exposed to DMEM-gent-DMSO only, set at 100%).

Statistical analysis

All experiments were performed multiple times with triplicate samples (see the legend for each Table or Figure). The data were expressed as mean ± standard deviation and were analyzed using a one-way analysis of variance and Scheffe's F procedure for *post hoc* comparisons, using STATVIEW® software (SAS Institute Inc., Cary, NC, USA).

Results

Cytotoxicity

Cytotoxic effects of simvastatin or atorvastatin were determined by measuring the ability of the fibroblasts to cleave the tetrazolium salt, MTT, to a formazan dye, and the results were expressed as a percentage of the control value (A_{595} in cells exposed to DMEM-gent only). Neither statin had significant cytotoxic effects after exposure times as long as 7 d (data not shown). There was no change in the cell morphology or in the pH of the media, and therefore media were not changed during the incubation period of this and of subsequent experiments (see below). In our previous studies, it was demonstrated that neither IL-1β nor LPS (1–50 μg/mL) was toxic to HGF (27,28).

Constitutive or IL-1β-stimulated production of RANKL and OPG

The production of OPG and RANKL by HGF was determined after incubation periods of 1–6 d, in the presence or absence of IL-1β. The cells produced lower constitutive levels of OPG (< 5 ng/mL) on days 1 and 3, which increased significantly in response to only the highest IL-1β concentration used (10 nM) (Fig. 1). After a 6-d period, constitutive OPG production was ~80 ng/mL, and this was generally stimulated by the higher IL-1β concentrations tested (0.1–10 nM) ($p = 0.001$) (Fig. 1). Constitutive or IL-1β-stimulated production of RANKL was in the pg/mL range, in contrast to the amounts of OPG produced under similar conditions. There was a trend towards increased production of

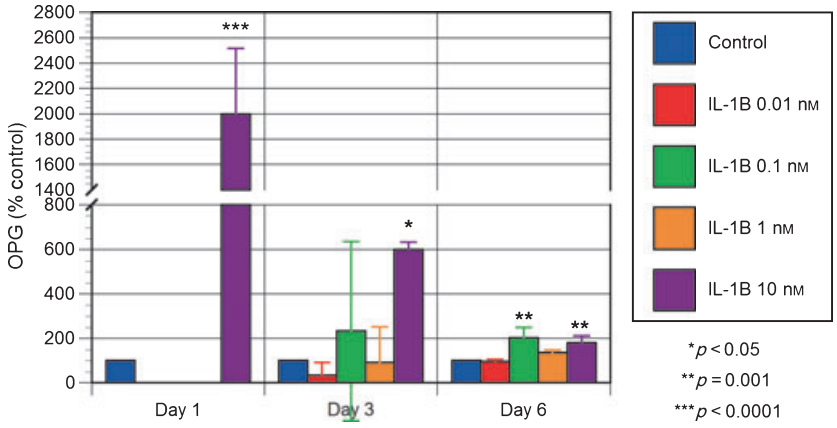

Fig. 1. Effect of interleukin-1 β (IL-1β) on the production of osteoprotegerin (OPG) from gingival fibroblasts. Fibroblasts were seeded in complete medium and cultured overnight at 37°C. The medium was removed, the cells were washed with phosphate-buffered saline and then serum-free medium supplemented with 100 μg/mL of gentamicin (DMEM-gent), with or without IL-1β (0.01–10 nM), was added. After 1, 3 or 6 d of incubation, the concentration of OPG in the cell supernatants was measured by ELISA. The results of two experiments, each with triplicate samples, were expressed as ng/mL in the cell supernatants and then converted to a percentage relative to the control value (i.e. the amount of OPG produced by cells exposed to DMEM-gent only, set at 100%).

RANKL with IL-1β stimulation, especially on days 3 and 6, but none of the changes were statistically significant (Fig. 2). Based on these experiments, the HGF were incubated for 6 d, in the presence or absence of 0.1 nM IL-1β, in experiments testing the effects of statins.

Effects of statins on constitutive or IL-1β-stimulated OPG production

Table 1 shows the effects of atorvastatin or simvastatin on constitutive or IL-1β-stimulated OPG production. With increasing concentration, there was a general trend for atorvastatin to elevate the constitutive production of OPG, particularly at concentrations between 1.0 and 100 nM. Simvastatin, on the other hand, had a minimal effect on OPG. In contrast, with increasing concentrations, atorvastatin showed a general trend of inhibiting IL-1β-stimulated OPG production. Similarly to its effects on constitutive OPG production, simvastatin had virtually no effect on IL-1β-stimulated OPG. None of the effects of either drug on OPG production was statistically significant.

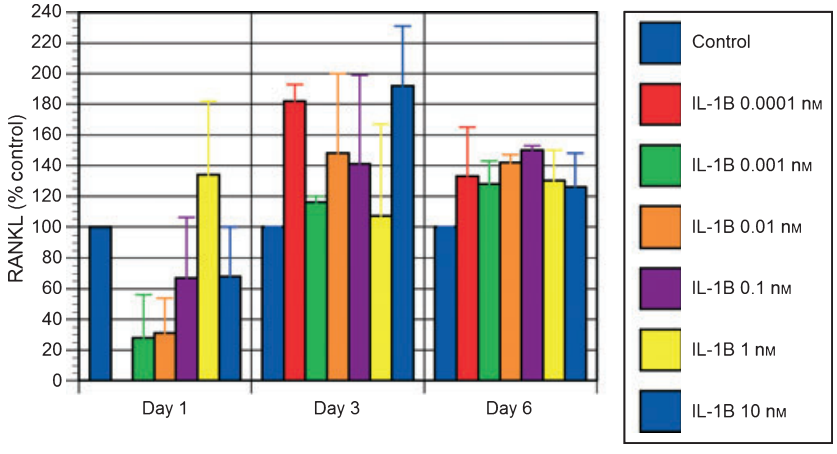

Fig. 2. Effect of interleukin-1 β (IL-1β) on the production of RANKL by gingival fibroblasts. Fibroblasts were seeded in complete medium and cultured overnight at 37°C. The medium was removed, the cells were washed with phosphate-buffered saline and then serum-free medium supplemented with 100 μg/mL of gentamicin (DMEM-gent), with or without IL-1β (0.0001–10 nM), was added. After incubation for 1, 3 or 6 d, the concentration of RANKL in the cell supernatants was measured by ELISA. The results of two experiments, each with triplicate samples, were first expressed as pg/mL in the cell supernatants and then converted to a percentage relative to the control value (i.e. the amount of RANKL produced by cells exposed to DMEM-gent only, set at 100%).

Effects of statins on constitutive or IL-1β-stimulated RANKL production

As shown in Table 2, both atorvastatin and simvastatin, in the range of 0.01 nM–5 μM, increased the constitutive production of RANKL. Atorvastatin had a more robust effect than simvastatin on constitutive RANKL production, especially at 0.01 and 0.1 nM. However, none of these increases reached statistical significance. Most concentrations of atorvastatin also increased the IL-1β-stimulated production of RANKL (Table 2). In contrast, no concentration of simvastatin had any effect or decreased the production of IL-1β-stimulated RANKL. As was the case with OPG production, none of the effects of the drugs on constitutive or IL-1β-stimulated RANKL reached statistical significance.

Effects of statins on constitutive and IL-1β-stimulated RANKL/OPG ratios

Both atorvastatin and simvastatin generally increased the constitutive RANKL/OPG ratios (Fig. 3), particularly at higher concentrations. The increases reached statistical significance at several drug concentrations: simvastatin at ≥ 10 nM and atorvastatin at ≥ 1 μM ($p \leq 0.01$). In contrast, simvastatin had no effect or decreased IL-1β-stimulated RANKL/OPG ratios, but none of the decreases were statistically significant (Fig. 4). Most lower concentrations of atorvastatin had no significant effect on RANKL/OPG ratios (~1.1–1.3 fold increase) in the presence of IL-1β. Only atorvastatin at 5 μM significantly increased the

Table 1. Effect of statins on constitutive and interleukin-1β (IL-1β)-stimulated osteoprotegerin (OPG) production by human gingival fibroblasts

	Constitutive OPG		IL-1β-stimulated OPG	
Statin concentration	A	S	A	S
0 (Control)	100 (0)	100 (0)	100 (0)	100 (0)
0.01 nM	143 (48)	112 (20)	113 (21)	105 (21)
0.1 nM	89 (20)	87 (8)	106 (34)	100 (9)
1 nM	106 (13)	118 (21)	91 (14)	100 (15)
10 nM	112 (21)	92 (12)	108 (30)	101 (10)
100 nM	132 (26)	95 (19)	102 (33)	103 (14)
1 μM	81 (19)	86 (14)	87 (10)	85 (15)
5 μM	102 (40)	122 (16)	72 (20)	102 (14)

Cells were pre-incubated with atorvastatin (A) or simvastatin (S) for 24 h and then incubated in the presence or absence of 0.1 nM IL-1β for 6 d. The concentration of OPG was measured (in ng/mL) and converted to a percentage relative to the control value (i.e. the amount produced by cells exposed only to DMEM-gent, set at 100%). The values represent mean (standard deviation), with the mean values representing the average of three experiments, each with triplicate samples.

Table 2. Effect of statins on constitutive and interleukin-1 β (IL-1β)-stimulated RANKL production by human gingival fibroblasts

	Constitutive RANKL		IL-1β-stimulated RANKL	
Statin concentration	A	S	A	S
0 (Control)	100 (0)	100 (0)	100 (0)	100 (0)
0.01 nM	213 (79)	134 (20)	123 (36)	94 (29)
0.1 nM	200 (78)	101 (6)	117 (16)	91 (20)
1 nM	174 (43)	131 (29)	108 (11)	87 (20)
10 nM	124 (20)	113 (19)	95 (10)	79 (22)
100 nM	194 (35)	152 (31)	91 (19)	84 (16)
1 μM	153 (38)	130 (31)	116 (18)	87 (33)
5 μM	192 (31)	164 (23)	116 (27)	101 (18)

Cells were pre-incubated with atorvastatin (A) or simvastatin (S) for 24 h, and then incubated in the presence or absence of 0.1 nM IL-1β for 6 d. RANKL was then measured (pg/mL) and converted to a percentage relative to the control value (i.e. the amount produced by cells exposed only to DMEM-gent, set at 100%). Values are averages of three experiments, each with triplicate samples. Figures in parentheses are standard deviations.

IL-1β-stimulated RANKL/OPG ratios (~1.6 fold increase; $p = 0.002$).

Discussion

This study examined the effect of simvastatin and atorvastatin on constitutive and IL-1β-stimulated production of OPG and RANKL by HGF. HGF comprise approximately two-thirds of the cell population in gingival tissue and are active participants in soft- and hard-tissue remodeling (29). In this study, the concentrations of simvastatin and atorvastatin ranged from 5×10^{-6} to 1×10^{-11} M, which is comparable to previous reports (5,7,8,30,31). There appears to be a large safety range of these drugs, when considering their effects on cells *in vitro*. Neither simvastatin nor atorvastatin, at any concentration tested, or at any time-point, was toxic to the HGF used in this study. These findings confirm those of other studies that reported a lack of toxicity of statins, in concentrations around the micromolar range or lower, to a variety of cell types. For example, Kamio *et al*. (32) found that simvastatin at 0.5 μM and atorvastatin at 2.5 μM did not affect the viability of human lung fibroblasts, and simvastatin at ≤ 1 μM had minimal toxicity to rat myocytes, using a mitochondrial dehydrogenase test similar to the one used in our study (33). Massaro *et al*. (34) also found that 10 μM atorvastatin or simvastatin did not affect the MTT activity in human umbilical vein endothelial cells after 12 h of exposure.

Our results showed that IL-1β was a potent stimulant of OPG by HGF on days 1, 3 and 6 for the highest dose (10 nM) and on day 6 for a lower dose (0.1 nM) (Fig. 1). Interestingly, IL-1β did not induce a significant increase in the production of RANKL by HGF (Fig. 2). Previous *in vitro* studies have used IL-1β to activate HGF, resulting in increased expression and production of cytokines, chemokines, transcription factors and adhesion molecules (35,36). Treatment of HGF with IL-1β has been utilized as an *in vitro* model to dissect the mechanistic pathways operable during the activation of HGF. Therefore, this *in vitro* model suggests that HGF treated with IL-1β produce an environment enriched for fibroblast OPG that attenuates bone resorption.

The effects of either statin on constitutive or IL-1β-stimulated production of OPG and RANKL did not reach significance at any concentration (Tables 1 and 2). However, both statins increased the constitutive RANKL/OPG ratio at multiple concentrations (Fig. 3), but only the high dose of atorvastatin (Fig. 4) induced a significant increase in the IL-1β-stimulated RANKL/OPG ratio. Therefore, within this particular model, the combination of statins and IL-1β treatment would appear to induce an environment that does not favor fibroblast contributions to bone resorption, compared with the effects of statins alone.

Several studies have explored the use of statins in the treatment of osseous defects or periodontitis-induced bone loss in animal models (37–39). It was reported that rats treated with simvastatin exhibited increased mandibular bone growth as well as a protective effect on tooth attachment and alveolar bone (37,38). Also, animals given local application of simvastatin following tooth extraction displayed newly formed bone islands and elevated

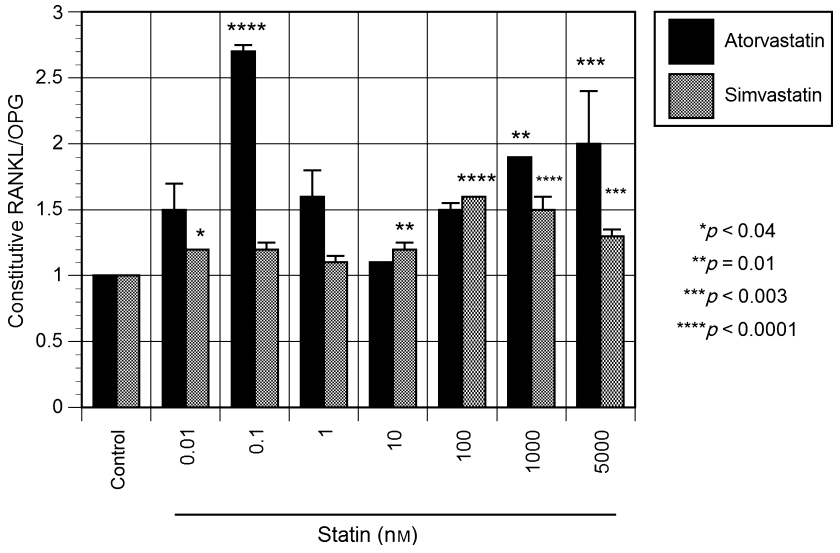

Fig. 3. Effect of statins on constitutive RANKL/osteoprotegerin (OPG) ratios. Fibroblasts were seeded in complete medium and cultured overnight at 37°C. The medium was removed, the cells were washed with phosphate-buffered saline and then serum-free medium supplemented with 100 μg/mL of gentamicin (DMEM-gent), with or without atorvastatin or simvastatin (0.01 nM–5 μM), was added. After 6 d of incubation, the concentrations of RANKL and OPG in the cell supernatants were measured by ELISA. The results of three experiments, each with triplicate samples, were expressed as ng/mL in the cell supernatants, and the ratios were calculated.

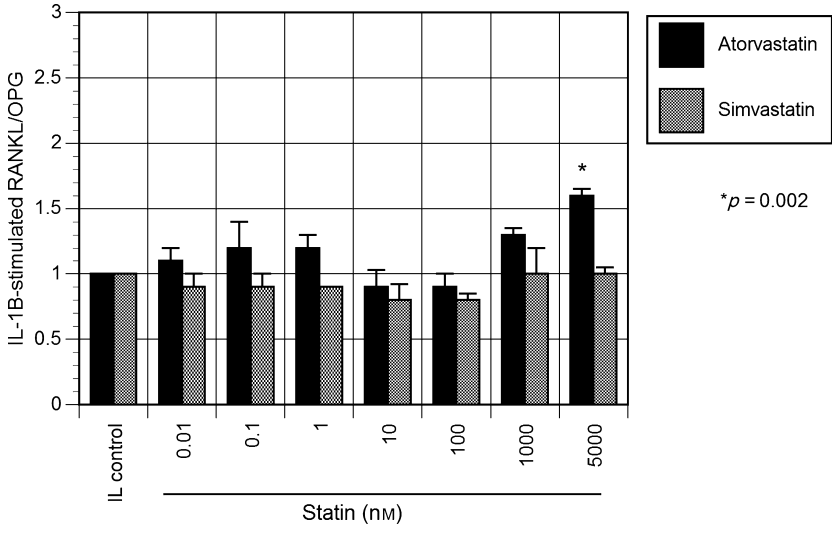

Fig. 4. Effect of statins on interleukin-1 β (IL-1β)-stimulated RANKL/osteoprotegerin (OPG) ratios. Fibroblasts were seeded in complete medium and cultured overnight at 37°C. The medium was removed, the cells were washed with phosphate-buffered saline and then serum-free medium supplemented with 100 μg/mL of gentamicin (DMEM-gent), with or without atorvastatin or simvastatin (0.01 nM–5 μM), was added. After 24 h of incubation with the statins, IL-1β (final concentration 0.1 nM) was added. After 6 d of incubation, the concentrations of RANKL and OPG were measured in the cell supernatants by ELISA. The results of three experiments, each with triplicate samples, were expressed as ng/mL in the cell supernatants, and the ratios were calculated.

bone-formation rates compared with untreated controls (39). Retrospective studies have shown that patients with advanced chronic periodontitis treated with simvastatin or atorvastatin had fewer periodontal lesions when measured using a novel index – the periodontal inflammatory burden index (40). Conversely, Saxlin *et al.* (41) reported that among subjects with no gingival bleeding, statin medication was associated with an increased likelihood of having teeth with deepened periodontal pockets. Two recently completed prospective studies support the role of statins as an adjunctive therapeutic in the treatment of periodontitis. Pradeep & Thorat (42) demonstrated a greater decrease in the gingival index and in pocket depth with a significant intrabony defect fill at sites treated with scaling and root planing plus a subgingivally delivered simvastatin gel. Fajardo *et al.* (43) reported that periodontitis subjects receiving scaling and root planing, in combination with 20 mg of daily atorvastatin, exhibited a significant improvement in alveolar bone height and diminished tooth mobility compared with patients who received only periodontal instrumentation.

This review of the periodontal literature on the effects of statins offers some mixed results. Prospective studies support the role of statins as an adjunctive therapeutic, in combination with scaling and root planing, for the treatment of chronic periodontitis. Simvastatin has been successfully utilized when delivered as a subgingival gel, and an oral daily dose of atorvastatin has also proven beneficial. However, the study by Saxlin *et al.* (41) offers some potential issues to consider. The beneficial effects of statin medication on the periodontium was only apparent in subjects with elevated levels of dental plaque or gingival bleeding. Among subjects with minimal gingival bleeding or dental plaque, statin medication was associated with an increased likelihood of having teeth with deepened periodontal pockets. Their conclusion was that the effect of statins upon the periodontium was dependent on the inflammatory condition of the gingival tissue. When viewed through this prism, our finding that either statin increased the constitutive RANKL/OPG ratio (Fig. 3), but minimally altered the IL-1β-stimulated ratio in HGF (Fig. 4), supports their observations. These results support the

potential for utilizing statins as an adjunctive therapeutic during initial instrumentation, when gingival inflammation is routinely encountered.

The results of this study are only applicable to the HGF cell line used and should not be utilized to draw general conclusions about the heterogeneous population of fibroblasts present in the periodontium. It should be emphasized that the results of the study support the conclusion from previous investigators regarding the osteoprotective properties of HGF based upon their elevated constitutive production of OPG (in the ng range) compared with RANKL (in the pg range) and confirm their role in the remodeling of osseous tissue. Both statins increased the constitutive ratio of RANKL/OPG in HGF. Additional studies are needed to further elucidate the effect of statins on the production of OPG and RANKL by HGF, in the presence or absence of other inflammatory mediators associated with chronic periodontitis.

Acknowledgements

We thank the University of Tennessee College of Dentistry Alumni foundation for their support. We also thank the Southern Academy of Periodontology for recognizing Dr Eva Dean in the Billy M. Pennel Student Research competition. The authors report no conflict of interest.

References

1. Maron JD, Fazio S, Linton FM. Current perspectives on statins. *Circulation* 2000; **101:**207–213.
2. Roche VF. Teachers topics:antihyperlipidemic statins. A self-contained clinically relevant medicinal chemistry. lesson. *Am J Pharm Educ* 2005;**4:**546–560.
3. Mora S, Ridker PM. Justification for the use of statins in primary prevention: an intervention trial evaluating rosavastatin (JUPITER)-can C-reactive protein be used to target statin therapy in primary prevention? *Am J Cardiol* 2006;**97**(2A)**:** 33A–41A.
4. Ridker PM, Danielson E, Fonseca FAH *et al*. Rosuvastatin to prevent vascular events in men and women with elevated C-reactive protein. *N Engl J Med* 2008; **359:**2195–2207.
5. Mundy G, Garett R, Harris S *et al*. Stimulation of bone formation in vitro and in rodents by statins. *Science* 1999;**286:**1946–1949.
6. Mundy G, Boyce B, Hughes D *et al*. The effect of cytokines and growth factors on osteoblastic cells. *Bone* 1995;**17:**1771–1775.
7. Yazawa H, Zimmermann B, Yoshiko A, Bernimoulin JP. Simvastatin promotes cell metabolism, proliferation and osteoblastic differentiation in human periodontal ligament cells. *J Periodontol* 2005;**76:**295–299.
8. Vierecek V, Grundker C, Blaschke S *et al*. Atorvastatin stimulates the production of osteoprotegerin by human osteoblasts. *J Cell Biochem* 2005;**96:**1244–1253.
9. Istvan ES, Deisenhofer J. Structural mechanism for statin inhibition of HMG-CoA reductase. *Science* 2001;**292:**1160–1164.
10. Boyle WJ, Simonet WS, Lacey DL. Osteoclast differentiation and activation. *Nature* 2003;**5:**337–342.
11. Horiuchi N, Maeda T. Statins and bone metabolism. *Oral Dis* 2006;**2:**85–101.
12. Ranney RR. Immunologic mechanisms of pathogenesis in periodontal diseases: an assessment. *J Periodontal Res* 1991;**26:** 243–254.
13. Offenbacher S. Periodontal disease: pathogenesis. *Ann Periodontol* 1996;**1:**821–878.
14. Kornman KS, di Giovine FS. Genetic variations in cytokine expression: a risk factor for severity of adult periodontitis. *Ann Periodontol* 1998;**3:**327–338.
15. Horton JE, Raisz LG, Simmons HA. Bone resorbing activity in supernatant fluid from culture peripheral blood leukocytes. *Science* 1972;**177:**793–795.
16. Hsu H, Lacey DL, Dunstan CR, Solovyev I, Colombero A, Timms E. Tumor necrosis factor receptor family member RANK mediates osteoclast differentiation and activation induced by osteoprotegerin ligand. *Proc Natl Acad Sci U S A* 1999;**96:** 3540–3545.
17. Lacey DL, Timms E, Tan HL, Kelley MJ, Dunstan CR, Burgess T. Osteoprotegerin is a cytokine that regulates osteoclast differentiation and activation. *Cell* 1998;**93:** 165–176.
18. Lerner UH. New molecules in the tumor necrosis factor ligand and receptor superfamilies with importance for physiologic and pathologic bone resorption. *Crit Rev Oral Biol Med* 2004;**15:**64–81.
19. Lerner UH. Inflammation-induced bone remodeling in periodontal disease and the influence of post-menopausal osteoporosis. *J Dent Res* 2006;**85:**596–607.
20. Bostanci N, Ilgenli T, Emingil G *et al*. Gingival crevicular fluid levels of RANKL and OPG in periodontal idseases: implications of their relative ratios. *J Clin Periodontol* 2007;**34:**370–376.
21. Jin Q, Cirelli J, Park C *et al*. RANKL inhibition through osteoprotegerin blocks bone loss in experimental periodontitis. *J Periodontol* 2007;**78:**1300–1308.
22. Hormdee D, Nagasawa T, Kiji M *et al*. Protein kinase-A-dependent osteoprotegerin production on IL-1 stimulation in human gingival fibroblasts is distinct from periodontal ligament fibroblasts. *Clin Exp Immunol* 2005;**142:**490–497.
23. Nagasawa T, Kobayashi H, Kiji M *et al*. LPS-stimulated human gingival fibroblasts inhibit the differentiation of monocytes into osteoclasts through the production of osteoprotegerin. *Clin Exp Immunol* 2002;**130:**338–344.
24. Kiji M, Nagasawa T, Hormdee D *et al*. Internal prostaglandin synthesis augments osteoprotegerin production in human gingival fibroblasts stimulated by lipopolysaccharide. *Clin Exp Immunol* 2007; **149:**327–334.
25. Belibasakis GN, Bostanci N, Hashim A *et al*. Regulation of RANKL and OPG gene expression in human gingival fibroblasts and periodontal ligament cells by *Porphyromonas gingivalis*: a putative role of the Arg-gingipains. *Microb Pathog* 2007;**43:**71–76.
26. Tipton DA, Pabst MJ, Dabbous Mkh. Interleukin-1β and tumor necrosis factorα independent monocyte stimulation of fibroblast collagenase activity. *J Cell Biochem* 1990;**44:**253–264.
27. Tipton DA, Seshul BS, Dabbous MKh. Effect of bisphosphonates on the production of mediators of osteoclastogenesis (RANKL, OPG, and IL-6) by human gingival fibroblasts. *J Periodontal Res* 2011;**46:**39–47.
28. Tipton DA, Flynn JC, Stein SH, Dabbous MKh. COX-2 inhibitors decrease IL-1β stimulated PGE_2 and IL-6 production by human gingival fibroblasts. *J Periodontol* 2003;**74:**1754–1763.
29. Lindhe J, Karring T, Lang PN. *Clinical Periodontology and Implant Dentistry*, 4th edition. Copenhagen: Blackwell Munksgaard, 2003.
30. Cohen E, Hohensinner P, Kaun C *et al*. Statins decrease TNF-α-induced osteoprotegerin production by endothelial cells and smooth muscle cells in vitro. *Biochem Pharmacol* 2007;**73:**77–83.
31. Martin J, Denver R, Bailey M, Krum H. In vitro inhibitory effects of atorvastatin on cardiac fibroblasts: implications for ventricular remodelling. *Clin Exp Pharmacol Physiol* 2005;**32:**697–701.
32. Kamio K, Liu X, Sugiura H *et al*. Statins inhibit matrix metalloproteinase release from human lung fibroblasts. *Eur Respir J* 2010;**35:**637–646.
33. Itagaki M, Takaguri A, Kano S *et al*. Possible mechanisms underlying statin-induced skeletal muscle toxicity in L6

fibroblasts and in rats. *J Pharmacol Sci* 2009;**109:**94–101.
34. Massaro M, Zampoli A, Soditti E *et al*. Statins inhibit cyclooxygenase-2 and matrix metalloproteinase-9 in human endothelial cells: anti-angiogenic actions possibility contributing to plaque stability. *Cardiovasc Res* 2010;**86:**311–320.
35. Vardar-Sengal S, Arora S, Baylas H, Mercola D. Expression profile of human gingival fibroblasts induced by IL-1β reveals central role of nuclear factor-kappa B in stabilizing human gingival fibroblasts during inflammation. *J Periodontol* 2009; **80:**833–849.
36. Agarwal S, Baran C, Piesco NP *et al*. Synthesis of proinflammatory cytokines by human gingival fibroblasts in reponse to lipopolysaccharides and IL-1β. *J Periodontal Res* 1995;**30:**382–389.
37. Stein D, Lee Y, Schmid MJ *et al*. Local simvastatin effects on mandibular bone growth and inflammation. *J Periodontol* 2005;**76:**1861–1870.
38. Vaziri H, Naserhojjati-Roodsari R, Tahsili-Fahadan N *et al*. Effect of simvastatin administration on periodontitis-associated bone loss in ovariectomized rats. *J Periodontol* 2007;**78:**1561–1567.
39. Wu Z, Liu C, Zang G, Sun H. The effect simvastatin on remodeling of the alveolar bone following tooth extraction. *Int J of Oral Maxillofac Surg* 2008;**37:**170–176.
40. Lindy O, Suomalainen K, Makela M, Lindy S. Statin use is associated with fewer periodontal lesions: a retrospective study. *BMC Oral Health* 2008;**8:**16–22.
41. Saxlin T, Suominen-Taipale L, Knuuttila M, Alha P, Ylostalo P. Dual effect of statin medication on the periodontium. *J Clin Periodontol* 2009;**36:**997–1003.
42. Pradeep AR, Thorat MS. Clinical effects of subgingivally delivered simvastatin in the treatment of patients with chronic periodontitis: a randomized clinical trial. *J Periodontol* 2010;**81:**214–222.
43. Fajardo ME, Rocha ML, Sanchez-Marin FJ, Espinosa-Chavez EJ. Effect of atorvastatin on chronic periodontitis: a randomized pilot study. *J Clin Periodontol* 2010;**37:**1016–1022.