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Statins Regulate IL-1β - Induced RANKL and OPG Production by Human Gingival Fibroblasts

Abstract

Three-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase competitive inhibitors, also known as statins, are widely used agents for lowering cholesterol and reducing the risk for a heart attack. Recent data suggest that statins influence bone metabolic activity by stimulating new bone formation both in vivo and in vitro. Bone resorption in periodontitis, an inflammatory disease, is orchestrated by the interaction of various cytokines in the inflamed tissue, produced by immune cells and also by resident cells such as human gingival fibroblasts (HGF) and periodontal ligament (PDL) cells. Three molecules, members of the tumor necrosis factor (TNF) ligand and receptor superfamilies, regulate the process of osteoclast formation. The first one, receptor activator for NF- kB ligand (RANKL) is expressed on hematopoetic stromal cells and periosteal osteoblasts as well as on HGF and PDL cells. RANKL interacts with its corresponding receptor, RANK, on mononucleated osteoclast precursors and induces their activation to multinuclear bone resorbing osteoclasts. The effects of RANKL are blocked by its soluble decoy receptor, osteoprotegerin (OPG), thus inhibiting osteoclast differentiation, activation and survival. RANKL and OPG are produced by many cells in the body including human gingival fibroblasts and PDL cells. At the moment, no data exist comparing the effect of statins on OPG/ RANKL production by resting and interleukin-1β (IL-1β)-stimulated HGF. Objective: The purpose of this project was to evaluate OPG and RANKL production in resting and IL-1β-stimulated HGFs, and to determine the effect of statins on their production. Methods: Cytotoxicity of statins was determined using an assay that measures the activity of a mitochondrial enzyme. Fibroblasts were preincubated with atorvastatin or simvastatin for 24 hours in serum-free medium, and then incubated without a stimulus or with IL-1β for 6 days. OPG or RANKL in culture supernatants was measured by specific ELISA. Data were analyzed using ANOVA and Scheffe's F procedure for post hoc comparison. Results: Concentrations of simvastatin or atorvastatin (5×10^{-6} M to 1×10^{-11} M) had no significant effect on the viability of the fibroblasts, after 7 days of exposure. IL-1β (1×10^{-8} M) increased OPG production significantly at day 1, 3 and 6, while IL-1β (1×10^{-10} M) increased OPG production significantly only on day 6. There was a trend towards increasing RANKL production with IL-1β stimulation, but no statistical significance was reached. When they had an effect, the statins tended to increase constitutive OPG and RANKL production and to decrease it in the presence of IL-1β, but these findings were not statistically significant. Both statins significantly increased the constitutive RANKL/OPG ratio at multiple concentrations. At the highest concentration (5×10^{-6} M), atorvastatin significantly increased the IL-1β stimulated RANKL/OPG ratio. Conclusion: Under IL-1β stimulation and in the absence of statins, OPG production by HGFs was increased significantly. Simvastatin and atorvastatin differed minimally in their effects on OPG and RANKL production by resting and IL-1β-activated HGFs. Both statins increased constitutive RANKL/OPG ratios, a finding suggesting that in the absence of inflammation statins may influence the production of RANKL and OPG by HGFs to favor bone catabolism.

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STATINS REGULATE IL-1β - INDUCED RANKL AND OPG PRODUCTION BY HUMAN GINGIVAL FIBROBLASTS

A Thesis
Presented for
The Graduate Studies Council
The University of Tennessee
Health Science Center

In Partial Fulfillment
Of the Requirements for the Degree
Master of Dental Science
From The University of Tennessee

By
Ivelina N. Jurkowski, D.M.D.
May 2010

DEDICATION

To John and Hannah Dean, for all the encouragement and unconditional love.

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ABSTRACT

Three-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase competitive inhibitors, also known as statins, are widely used agents for lowering cholesterol and reducing the risk for a heart attack. Recent data suggest that statins influence bone metabolic activity by stimulating new bone formation both in vivo and in vitro. Bone resorption in periodontitis, an inflammatory disease, is orchestrated by the interaction of various cytokines in the inflamed tissue, produced by immune cells and also by resident cells such as human gingival fibroblasts (HGF) and periodontal ligament (PDL) cells. Three molecules, members of the tumor necrosis factor (TNF) ligand and receptor superfamilies, regulate the process of osteoclast formation. The first one, receptor activator for NF- κB ligand (RANKL) is expressed on hematopoetic stromal cells and periosteal osteoblasts as well as on HGF and PDL cells. RANKL interacts with its corresponding receptor, RANK, on mononucleated osteoclast precursors and induces their activation to multinuclear bone resorbing osteoclasts. The effects of RANKL are blocked by its soluble decoy receptor, osteoprotegerin (OPG), thus inhibiting osteoclast differentiation, activation and survival. RANKL and OPG are produced by many cells in the body including human gingival fibroblasts and PDL cells. At the moment, no data exist comparing the effect of statins on OPG/ RANKL production by resting and interleukin-1β (IL-1β)-stimulated HGF. **Objective**: The purpose of this project was to evaluate OPG and RANKL production in resting and IL-1β-stimulated HGFs, and to determine the effect of statins on their production. **Methods:** Cytotoxicity of statins was determined using an assay that measures the activity of a mitochondrial enzyme. Fibroblasts were preincubated with atorvastatin or simvastatin for 24 hours in serum-free medium, and then incubated without a stimulus or with IL-1β for 6 days. OPG or RANKL in culture supernatants was measured by specific ELISA. Data were analyzed using ANOVA and Scheffe's F procedure for post hoc comparison. **Results:** Concentrations of simvastatin or atorvastatin (5×10^{-6} M to 1×10^{-11} M) had no significant effect on the viability of the fibroblasts, after 7 days of exposure. IL-1β (1×10^{-8} M) increased OPG production significantly at day 1, 3 and 6, while IL-1β (1×10^{-10} M) increased OPG production significantly only on day 6. There was a trend towards increasing RANKL production with IL-1β stimulation, but no statistical significance was reached. When they had an effect, the statins tended to increase constitutive OPG and RANKL production and to decrease it in the presence of IL-1β, but these findings were not statistically significant. Both statins significantly increased the constitutive RANKL/OPG ratio at multiple concentrations. At the highest concentration (5×10^{-6} M), atorvastatin significantly increased the IL-1β stimulated RANKL/OPG ratio. **Conclusion:** Under IL-1β stimulation and in the absence of statins, OPG production by HGFs was increased significantly. Simvastatin and atorvastatin differed minimally in their effects on OPG and RANKL production by resting and IL-1β-activated HGFs. Both statins increased constitutive RANKL/OPG ratios, a finding suggesting that in the absence of inflammation statins may influence the production of RANKL and OPG by HGFs to favor bone catabolism.

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CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW

Three-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) competitive inhibitors, also known as statins, are widely used agents for lowering cholesterol and reducing the risk for a heart attack (1). They lower cholesterol by inhibiting the enzyme HMG-CoA reductase (HMGR), which is the rate-limiting enzyme of the mevalonate pathway of cholesterol synthesis (Figure 1). Inhibition of this enzyme in the liver results in decreased cholesterol synthesis as well as increased synthesis of low-density lipoprotein (LDL) receptors, resulting in an increased clearance of low-density lipoprotein from the bloodstream (1). The first results can be seen after one week of use and the effect is maximal after four to six weeks (2).

In the 1970s the Japanese microbiologist Akira Endo first discovered natural products with a powerful inhibitory effect on HMGR in a fermentation broth of *Penicillium citrinum*, during his search for antimicrobial agents (3). The first product was named compactin (ML236B or mevastatin). In 1978, Alfred Alberts and colleagues discovered a new natural product in a fermentation broth of *Aspergillus terreus*, which showed good HMG-CoA reductase inhibition. They named the product mevinolin, which later became known as lovastatin (Figure 2) (4). The "oyster mushroom" (Figure 3), a culinary mushroom, naturally contains up to 2.8% lovastatin on dry weight basis (5).

Thus, statins have been on the market for over 20 years and appear to have relatively good safety profiles (6). Muscle symptoms, ranging from mild myalgia to clinically important rhabdomyolysis, are an important side effect of these drugs and are the leading cause of non-compliance. However, some patients may be susceptible to statin myopathy because of pre-existing subclinical inherited muscular disorders, genetic variations in statin uptake proteins or variations in genes affecting pain perception (7). Statin use grew from 47% of all lipid-lowering medications in 1992 to 87% in 2002, with atorvastatin being the leading medication. Currently about 13 million people in the United States take statins to lower cholesterol levels and reduce the risk of heart disease (8). Statins differ in their mode of derivation as synthetic or fermentation products, lipophilicity, and potency. Examples of fermentation-derived statins are simvastatin, pravastatin and lovastatin, while atorvastatin, cerivastatin, fluvastatin, pitavastatin and rosuvastatin are synthetic. The essential structural components of all statins are a dihydroxyheptanoic acid unit and a ring system with different substituents. The statin pharmacophore (Figure 4) is modified hydroxyglutaric acid component, which is structurally similar to the endogenous substrate HMG-CoA and the mevaldyl-CoA transition state intermediate (9).

The statins differ with respect to their ring structure and substituents (10). These differences in structure affect the pharmacological properties of the statins, such as: affinity for the active site of the HMGR, rates of entry into hepatic and non-hepatic tissues, availability in the systemic circulation for uptake into non-hepatic tissues, routes and modes of metabolic transformation and elimination. Statins have sometimes been

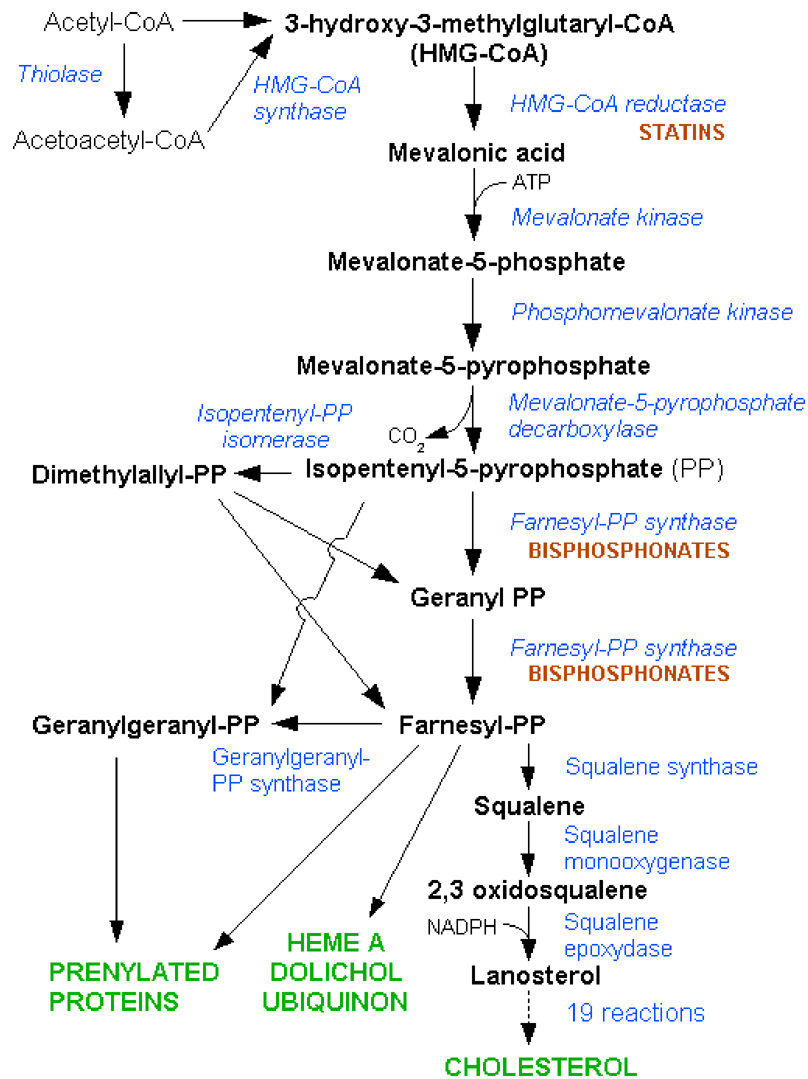

Figure 1. HMG-CoA reductase pathway.
Reprinted from *Wikipedia, The Free Encyclopedia.* Retrieved January 15, 2010, from http://en.wikipedia.org/wiki/File:HMG-CoA_reductase_pathway.png (10).

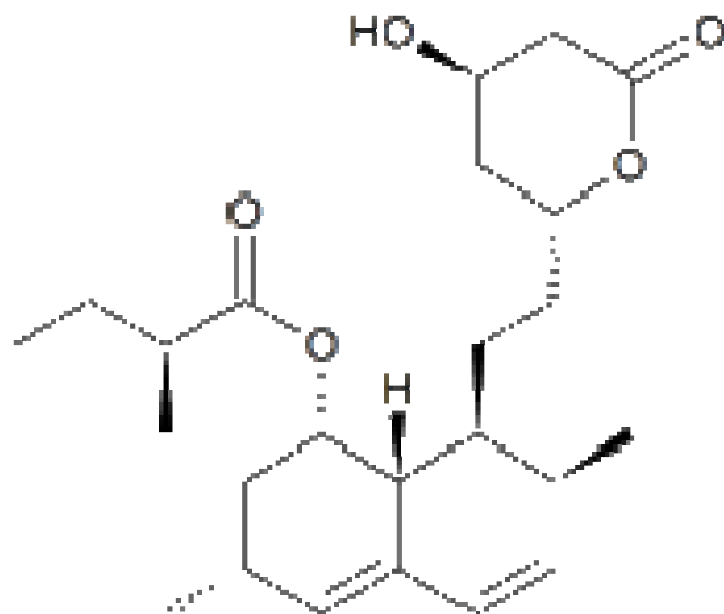

Figure 2. Lovastatin.
Reprinted from *Wikipedia, The Free Encyclopedia.* Retrieved January 15, 2010, from http://en.wikipedia.org/wiki/Lovastatin (11).

Figure 3. Oyster mushroom.
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http://en.wikipedia.org/wiki/File:Oyster_mushroom_log.jpg (12).

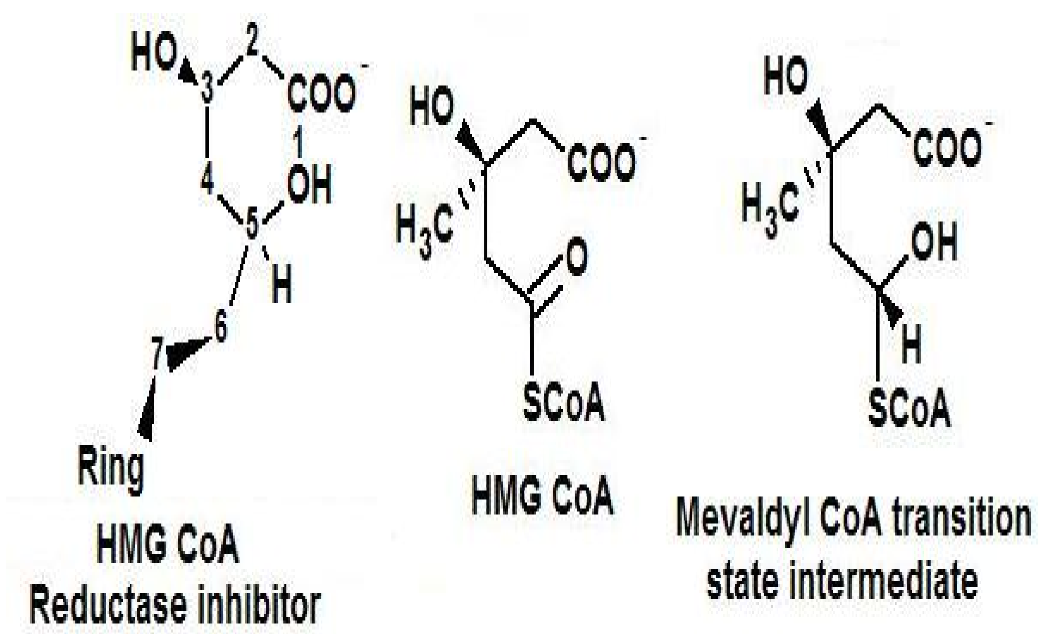

Figure 4. The statin pharmacophore.
Reprinted from *Wikipedia, The Free Encyclopedia.* Retrieved January 15, 2010, from http://en.wikipedia.org/wiki/Statin_development#The_statin_pharmacophore (13).

divided into two types according to their structure (14). Type 1 statins have substituted decalin-ring structure that resembles the first statin ever discovered, mevastatin. Statins that belong to this group are: lovastatin, simvastatin and pravastatin. Type 2 statins are fully synthetic and have larger groups linked to the HMG-like moiety. One of the main differences between the type 1 and type 2 statins is the replacement of the butyryl group of type 1 statins by the fluorophenyl group of type 2 statins. This group is responsible for additional polar interactions that cause tighter binding to the HMGR and relatively higher potency of this group as compared to type 1 statins in lowering LDL. Statins that belong to this group are: fluvastatin, atorvastatin, cerivastatin and rosuvastatin (14).

Mevalonate, the product of HMGR reaction, is the precursor not only of cholesterol, but also of many nonsteroidal isoprenoids vital for diverse cell functions. It appears that the inhibition of HMG-CoA reductase is responsible for numerous pleiotropic effects of statins such as decreasing smooth muscle cells migration and proliferation, stimulation of nitric oxide (NO) production, anti-apoptosis, and immunomodulation (14-17). Thus, in addition to their lipid-lowering effects, statins have shown beneficial effect in various systems: cardiovascular, immune, nervous and skeletal (18-21). Lately, statins have shown potential in anti-cancer therapy and as immunosuppressors in organ transplantation (22-24).

Recent data suggest that statins influence bone metabolic activity by stimulating new bone formation both in vivo and in vitro. Mundy et al. identified statins as a potent activator of bone morphogenic protein-2 (BMP-2), an important stimulator of osteoblastic differentiation (25). In vitro studies have shown that simvastatin, mevastatin and atorvastatin stimulated BMP-2 transcription and also increased endogenous BMP-2 mRNA and protein expression in human MG63 osteoblastic cells by two fold (26, 27). Other investigators have reported an increase in BMP-2 transcription at doses from 0.1 μM to 5 μM for lovastatin, simvastatin, mevastatin, and atorvastatin in cultures of murine neonatal calvarias. Cytotoxicity resulted from higher concentrations (28). Initial in vivo experiments with rodents showed that when injected locally over the calvarias of normal mice, statins increased calvarial width by 30-50% (25). Yazawa et al. reported stimulation of alkaline phosphate activity of human PDL cells by simvastatin, which was dose-dependent (29). Maximum effect was obtained at concentration of 1 μM. In addition to their ability to enhance osteoblastic differentiation, statins also stimulated the production of OPG, which may contribute to their bone-sparing effect (30). This effect is amplified by the blockage of mevalonate-derivatives formation such as farnesyl-pyrophosphate and geranyl-pyrophosphate, two agents necessary for activation of osteoclasts. Statins, by inhibiting the HMG-CoA reductase pathway, act to abate bone resorption (9).

These findings in the field of bone metabolism suggest that statins could be considered as potential agents for treating osteoporosis and other diseases of bone loss such as periodontal disease (31, 32). Both diseases share common pathogenic pathways. Up-regulation of intereukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) induces osteoclastic activity and increased bone turnover rates leading to bone mass loss and osteoporosis (34). In addition to their osteoprotective properties, statins

may be able to influence the amount of tissue destruction in periodontal disease by interacting with the host's immune response. It has been demonstrated that statins can inhibit leukocyte adhesion and extravasation in sites of inflammation. This in turn may result in impaired T-cell costimulation and decrease of T-cell inflammatory cytokines such as IL-1β, IL-6 and TNF-α as well as decreased expression on T-lymphocytes of molecules involved with bone metabolism (34, 35).

IL-1 exists in two isoforms, namely IL-1α and IL-1β, with similar biological activities (36). IL-1β is a potent multifunctional cytokine that is involved in host immune and inflammatory responses. The various biologic effects are due to its ability to activate a wide range of factors, such as matrix metalloproteinases, nitric oxide synthase, prostanoids and other cytokines (37). IL-1β has been established as the primary factor associated with the pathogenesis of periodontal disease (38-40). It was first named *osteoclast-activating factor* because of its role in osteoclast differentiation and bone resorption (40). During the inflammatory response, cytokines such as IL-1β stimulate the periosteal osteoblasts, altering expression levels of specific protein members of the TNF ligand and receptor superfamilies on the osteoblast surface.

Thus bone apposition and bone resorption in periodontitis are governed by the interaction of various stimulating and inhibiting cytokines in the inflamed tissue, 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 TNF ligand and receptor superfamilies, regulate the process of osteoclast formation via cell-to cell interactions (41). The first one, receptor activator for NF- κB ligand (RANKL) is expressed on hematopoetic stromal cells and periosteal osteoblasts as well as on fibroblasts, and T- and B-lymphocytes. RANKL is expressed on osteoblasts as 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 (42). The effects of RANKL are blocked by its soluble decoy receptor osteoprotegerin (OPG), thus inhibiting osteoclast differentiation, activation and survival (43). When OPG concentrations are high relative to RANKL expression, OPG binds RANKL, inhibiting RANK-RANKL interaction, which leads to reduced formation of osteoclasts and apoptosis of preexisting osteoclasts (44). This environment favors bone formation. In the opposite scenario, when OPG levels are low relative to RANKL levels, RANKL is available to bind RANK on osteoclast precursors and bone resorption is favored.

Since inflammation-induced bone resorption is a hallmark of periodontal disease, it is expected to find increased RANKL/OPG ratio in the gingival crevicular fluid (GCF) of affected individuals. Indeed, elevated RANKL/OPG ratios were reported by several investigators. Bostanci et al. evaluated OPG and RANKL levels in healthy and periodontal disease subjects (45). The subjects with periodontal disease were categorized as: gingivitis, chronic periodontitis, generalized aggressive periodontitis and chronic periodontitis subjects. GCF RANKL and OPG levels were oppositely regulated in the periodontitis groups, but not in the gingivitis group, resulting in an elevated RANKL/OPG ratio. This ratio was similar in all three periodontitis groups and may

therefore predict disease occurrence. Therefore, the inhibition of RANKL by OPG may represent an important therapeutic strategy for the prevention and treatment of periodontal disease.

This assumption was used by Jin et al. when creating an experimental periodontitis model in rats and evaluating the effect of RANKL inhibition by OPG on bone loss (46). A total of 32 rats were administered human OPG-Fc fusion protein (10 mg/kg) or vehicle by subcutaneous delivery twice weekly for 6 weeks. Biopsies were harvested after 3 and 6 weeks, and mandibles were evaluated by microcomputed tomography (microCT) and histology. Significant preservation of alveolar bone volume was observed among OPG-Fc-treated animals compared to the controls at weeks 3 and 6. Descriptive histology revealed that OPG-Fc significantly suppressed osteoclast surface area at the alveolar crest. The results support the hypothesis that RANKL inhibition by OPG may represent an important therapeutic strategy for the prevention of progressive alveolar bone loss.

RANKL and OPG are produced by many cells in the body including HGF and PDL cells (47, 48). Some studies report an increase in OPG production by HGF when stimulated by lipopolysaccharide (LPS) from *Aggregatibacter actinomycetemcomitans (A.a.)* and *Porphyromonas gingivalis (P.g.*), which appears to inhibit monocyte differentiation into osteoclasts (48, 49). Other studies report induced RANKL and reduced OPG mRNA expression by HGF resulting in an increased RANKL/OPG expression ratio by *P.g.* (50). Thus, the resident cells may play a major role in RANK/RANKL/OPG balance at connective tissue/bone interface in health and in diseased states. Modulation of their secretory activity may be useful in achieving the primary goal for periodontal therapy, namely prevention of further soft and hard tissue destruction and regeneration of lost structures.

While a limited number of studies have focused on OPG/RANKL production by HGF, no studies have systematically evaluated the effect of IL-1β or statins on HGF production of both OPG and RANKL. Therefore the purpose of this study is to evaluate OPG and RANKL production in resting and IL-1β-activated HGF in the presence and absence of statins.

Purpose of Study

The overall purpose of this project was to examine the effects of two different statins, simvastatin and atorvastatin, on RANKL and OPG production by cultured human gingival fibroblasts.

Hypothesis

Based on the differences in the mode of derivation and potency, the hypothesis tested was that simvastatin and atorvastatin differ in their effect on OPG and RANKL production in resting and IL-1β-activated human gingival fibroblasts.

Specific Aims

- Specific Aim 1**:** To quantify constitutive OPG and RANKL production by human gingival fibroblasts.
- Specific Aim 2**:** To quantify OPG and RANKL production by human gingival fibroblasts following IL-1β stimulation.
- Specific Aim 3**:** To determine the effect of atorvastatin and simvastatin on OPG and RANKL production in resting and IL-1β-stimulated HGF.

CHAPTER 2. MATERIALS AND METHODS

Human Gingival Fibroblasts

A normal human gingival fibroblast cell line, previously established and available for the study, was used in this work. The cell line was established from gingival explants using standard techniques. The gingival explants were minced using a scalpel and then washed with Dulbecco's Modified Eagle Medium (DMEM; Gibco; Grant Island, NY) supplemented with 10% (v/v) newborn calf serum (NCS; Gibco) and 100 μg/ml gentamicin (Sigma-Aldrich Co; St Louis, MO) (complete medium), supplemented with 2.5 μg/ml amphotericin B (Sigma-Aldrich Co; St Louis, MO), 100 U/ml penicillin (Sigma-Aldrich Co; St Louis, MO) and 100 μg/ml streptomycin. The tissue pieces were placed in 25 cm² flasks, allowed to adhere, and then incubated in complete medium at 37°C in a humidified atmosphere of 5% CO_2 in air until outgrowth of fibroblasts occurred (within two weeks). At the point of confluency in the 25 cm² flasks, the cells were removed from the flasks by a brief (5 min) treatment with trypsin (0.25%; Gibco), transferred to 75 cm² flasks, and routinely cultured in complete medium.

Statins and IL-1β

Atorvastatin was provided by Pfizer (Groton, CT) at no cost through a material transfer agreement. Simvastatin was purchased from Sigma–Aldrich. Both drugs are soluble in dimethyl sulfoxide (DMSO). 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 compared to other statins at equal concentration and incubation time. Human recombinant IL-1β was purchased from R&D Systems, (Minneapolis, MN).

Determination of Cytotoxic Effect of Statins on Human Gingival Fibroblasts

In order to determine non-cytotoxic doses of the statins for experimental use, the effects of the drugs on cell viability were measured by determining their effects on the ability of the cells 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). Cells (2.5×10^4) were exposed to DMEM containing 100 μg/ml gentamicin (DMEM-gent) in the presence or absence of simvastatin or atorvastatin (5×10^{-6} M to 1×10^{-11} M) (final DMSO concentration = 0.1%). After exposure time of 7 days, MTT was added to the cells. Purple formazan crystals produced from the MTT by metabolically active cells were solubilized and absorbance read at 540 nm. Results were expressed as percent of control (A_{540nm} in cells exposed to DMEM-gent-0.1% DMSO only).

Determination of Constitutive and IL-1β-stimulated Production of RANKL and OPG

To obtain conditioned media, the cells were seeded at 3 x10^5 in 6-well plates in complete medium and cultured overnight at 37°C. This medium was removed and the wells were washed with PBS. DMEM-gent with or without IL-1β was added. Experimental conditions varied by incubation time, and concentration of IL-1β. Production of RANKL and OPG was measured in fibroblast-conditioned media using the Human DuoSet OPG ELISA Development System (R&D Systems), and a RANKL ELISA (Pepro Tech, Inc.; Rocky Hill, NJ). The media were harvested at specific time points (i.e. 1-6 d), and samples were assayed using specific ELISA to measure the levels of OPG or RANKL, per the manufacturer's instructions. Absorbance values were read using a microtiter plate spectrophotometer, and the results were expressed as the concentration of OPG or RANKL (pg/ml).

Determination of the Effects of Statins on Constitutive and IL-1β-stimulated Production of RANKL and OPG

The initial step of this experiment was carried out as described in the preceding section. After the wells were washed with PBS, DMEM-gent (1.5 ml/well) with or without statins (5 x 10^{-6} M to 1 x 10^{-11} M) (final DMSO concentration = 0.1%) was added. After 24 hours IL-1β (1 x 10^{-10} M) was added. Previous work from this laboratory established the lack of toxicity of 1x 10^{-10} M IL-1β, which was used as a stimulus in this last part of the study. The media were harvested at day six. Samples were assayed using the specific ELISA to measure the levels of OPG or RANKL, per the manufacturer's instructions. Absorbance values were read using a microtiter plate spectrophotometer, and the results were expressed as the amount of OPG or RANKL (pg/ml). These results were converted to percent of control (amount made by cells exposed to DMEM-gent-DMSO only, set at 100%).

Statistical Analysis

All experiments were performed multiple times with triplicate samples. The data were expressed as mean ± standard deviation and were analyzed using a one-way analysis of variance (ANOVA) and Scheffe's F procedure for post hoc comparisons, using StatView® software.

CHAPTER 3. RESULTS

Cytotoxic Effect of Statins on Human Gingival Fibroblasts

Cytotoxic effects of simvastatin and atorvastatin were determined using the MTT assay, which measures the ability of the fibroblasts to cleave the tetrazolium salt MTT to a formazan dye. The results were expressed as percent of control (A_{540nm} in cells exposed to DMEM-gent-0.1% DMSO only). Concentrations of simvastatin or atorvastatin of 5 x 10^{-6} M to 1 x 10^{-11} M had no significant effect on the viability of the fibroblasts, after 7 days of exposure (Figure 5).

Effect of IL-1β on Constitutive OPG Production

Previous work in this laboratory established lack of toxicity of 1 x 10^{-10} M IL-1β on the cell line used. Incubation times ranged from one to six days. Statistically significant increase ($p<0.0001$) was seen on day 1 between control and 1 x 10^{-8} M IL-1β (Figure 6). The increase ($p=0.005$) persisted on day 3 at the same concentration. On day 6, there was statistically significant difference for two concentrations of IL-1β: 1 x 10^{-8} M and 1 x 10^{-10} M ($p=0.001$) (Figure 6). Constitutive OPG levels produced by HGF were in the ng/ml range, and following IL-1β stimulation, the levels were increased by as much as 10- fold.

The levels of OPG (and RANKL) for the rest of the experiments were first expressed as ng/ml or pg/ml in the cell supernatants and then converted to percent of the control (amount produced by cells not exposed to the drug), set at 100 percent.

Effect of IL-1β on Constitutive RANKL Production

Identical incubation times as described in previous experiments for OPG were used for evaluating the effect of IL-1β on constitutive RANKL production. Constitutive RANKL levels produced by HGF were in the pg/ml range. There was a trend towards increasing RANKL production with IL-1β stimulation, especially at days 3 and 6. Following IL-1β stimulation, the levels of RANKL were increased 1-2 fold. None of the changes were statistically significant by Scheffe's test (Figure 7).

Effect of Statins on Constitutive RANKL Production

Both drugs in concentration range of 5 x 10^{-6} M to 1 x 10^{-11} M increased constitutive production of RANKL (Figures 8 and 9). Atorvastatin had a more robust effect on constitutive RANKL production compared to simvastatin especially at 1 x 10^{-10} M and 1 x 10^{-11} M. No increase reached statistical significance.

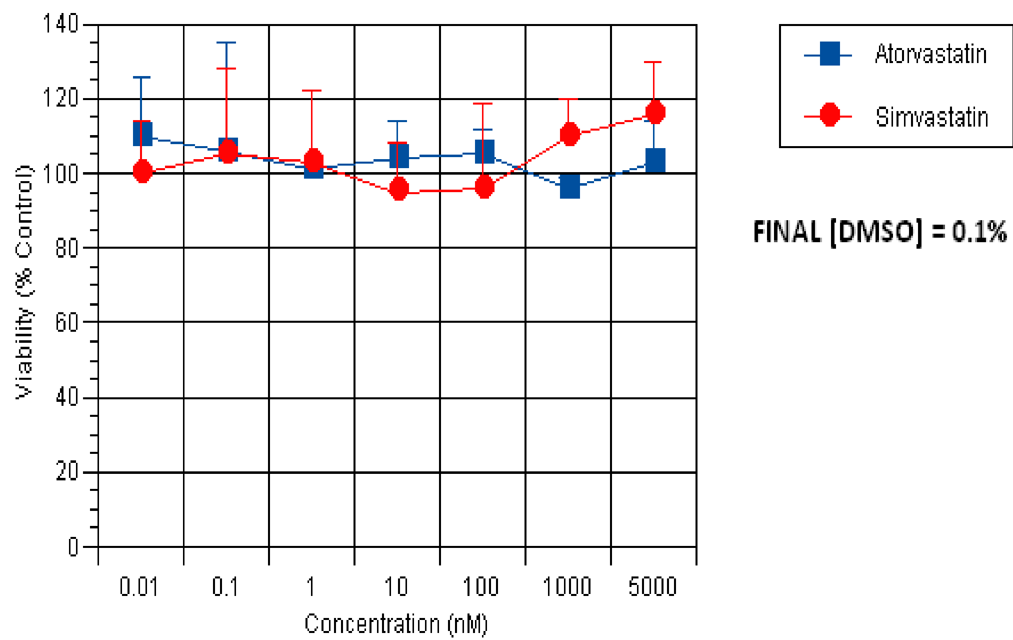

Figure 5. Cytotoxic effect of statins on human gingival fibroblasts.

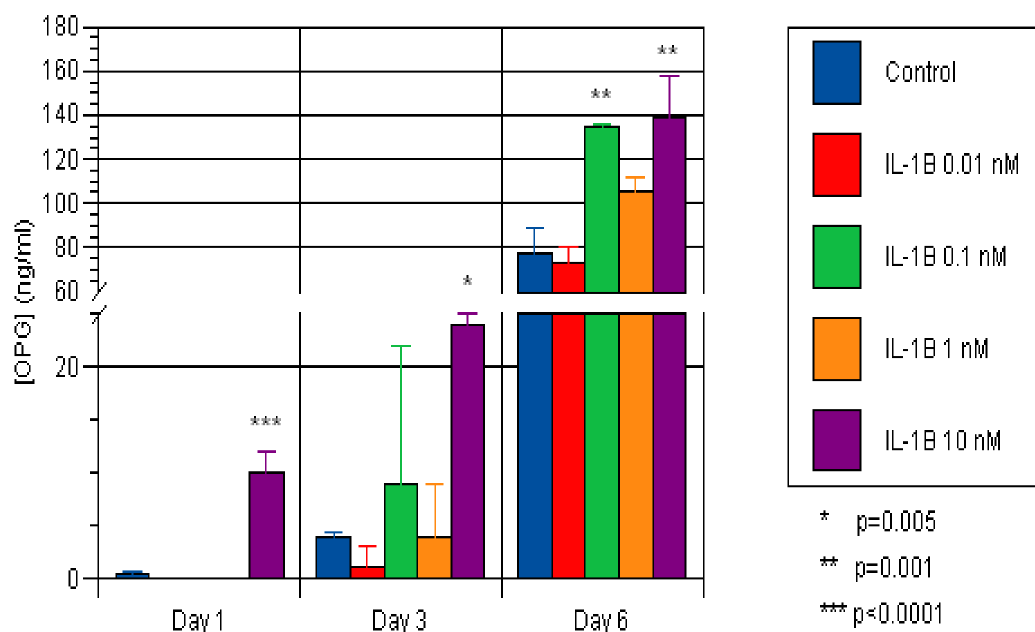

Figure 6. Effect of IL-1β on constitutive OPG production.

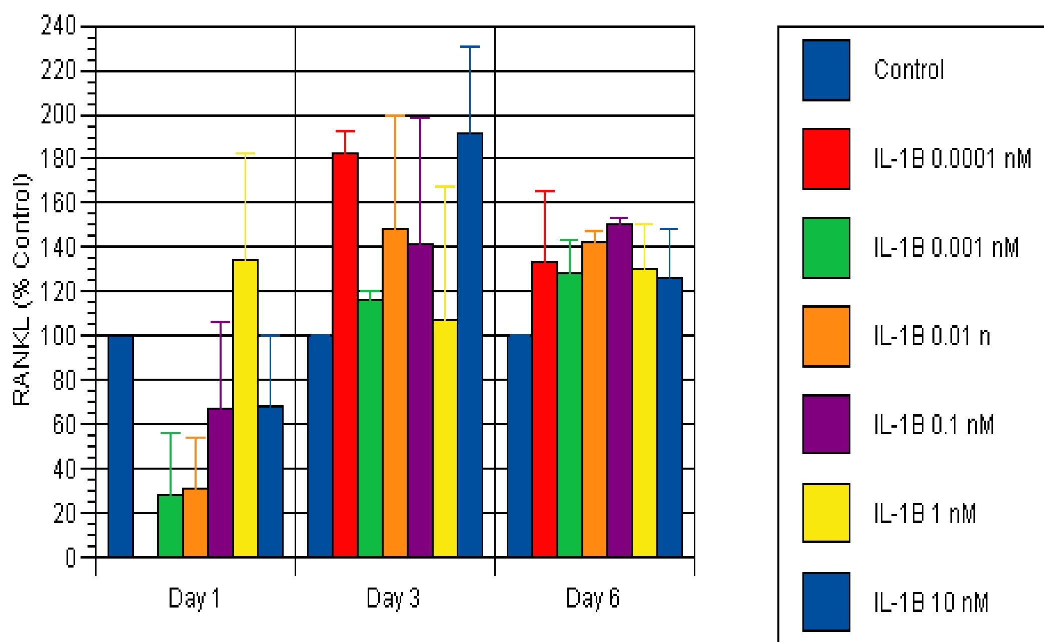

Figure 7. Effect of IL-1β on constitutive RANKL production.

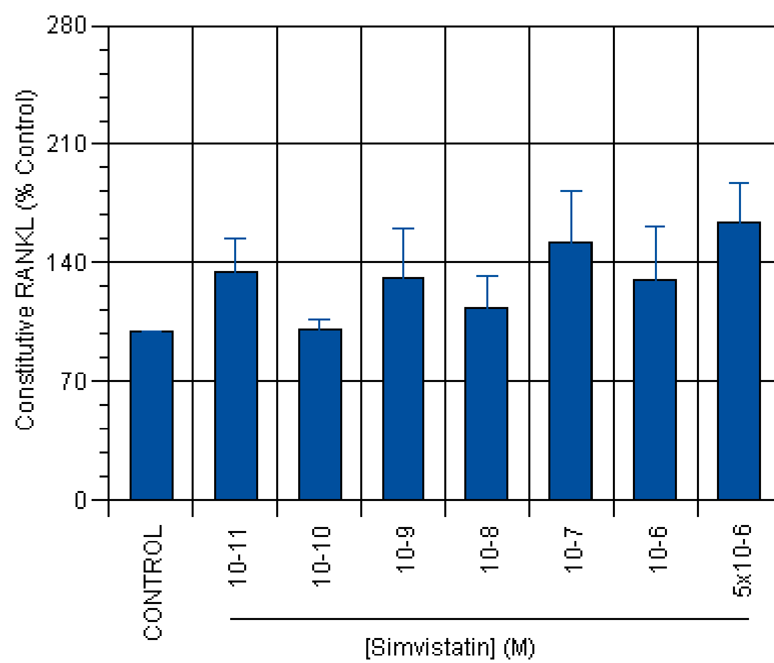

Figure 8. Effect of simvastatin on constitutive RANKL production.

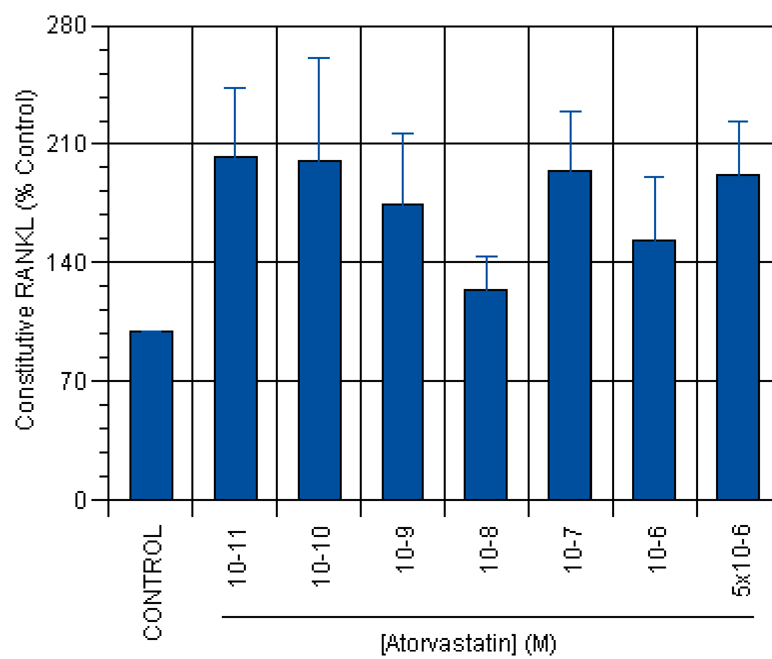

Figure 9. Effect of atorvastatin on constitutive RANKL production.

Effect of Statins on IL-1β-stimulated RANKL Production

All concentrations of simvastatin (except 5×10^{-6} M) decreased the IL-1β-stimulated production of RANKL. Maximum decrease was achieved for 1×10^{-8} M of simvastatin. All concentrations of atorvastatin (except 1×10^{-7} M and 1×10^{-8} M) increased the IL-1β-stimulated production of RANKL (Figures 10 and 11). Maximum increase in IL-1β stimulated RANKL was noticed again for 1×10^{-10} M and 1×10^{-11} M, while maximum decrease was achieved at 1×10^{-7} M of atorvastatin. None of the effects reached statistical significance.

Effect of Statins on Constitutive OPG Production

Simvastatin had a minimal effect on constitutive OPG production (Figure 12). Atorvastatin showed a trend towards increasing the constitutive production of OPG (Figure 13). The greatest increase was seen at 1×10^{-11} M. No statistical significance was detected for either drug for any concentration.

Effect of Statins on IL-1β-stimulated OPG Production

Most simvastatin concentrations had no effect on IL-1β stimulated production of OPG (Figure 14). A small decrease was noted at 1×10^{-6} M concentration. Atorvastatin showed a trend towards decreasing IL-1β-stimulated OPG production (Figure 15). However, decreases were noted at 1×10^{-6} M and 5×10^{-6} M, while the greatest increase in IL-1β-stimulated OPG production was seen at 1×10^{-11} M. Neither drug at any concentration produced a statistically significant change.

Effect of Statins on Constitutive RANKL/OPG Ratios

Both drugs in the concentrations tested increased the constitutive RANKL/OPG ratios (Figure 16). Statistical significance was detected by the Scheffe's test for several concentrations of atorvastatin (1×10^{-10} M, 1×10^{-6} M and 5×10^{-6} M) and simvastatin (1×10^{-11} M, 1×10^{-8} M, 1×10^{-7} M, 1×10^{-6} M and 5×10^{-6} M). Atorvastatin at 1×10^{-10} M ($p<0.0001$) produced 2.7 fold increase. Simvastatin at concentrations 1×10^{-11} M ($p<0.04$) and 1×10^{-8} M ($p=0.01$) produced the smallest increase.

Effect of Statins on IL-1β-stimulated RANKL/OPG Ratios

Most concentrations of simvastatin decreased IL-1β-stimulated RANKL/OPG ratios. The decrease was greatest at 1×10^{-7} M and 1×10^{-8} M, but did not reach statistical significance (Figure 17). Most concentrations of atorvastatin increased the ratio. There was a statistically significant increase ($p=0.002$) in the IL-1β-stimulated RANKL/OPG ratio at 5×10^{-6} M atorvastatin.

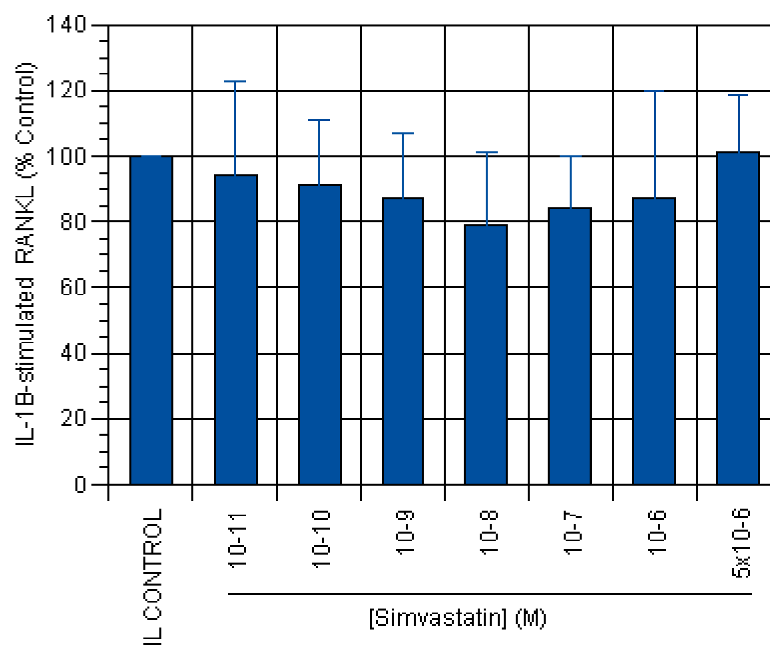

Figure 10. Effect of simvastatin on IL-1β-stimulated RANKL production.

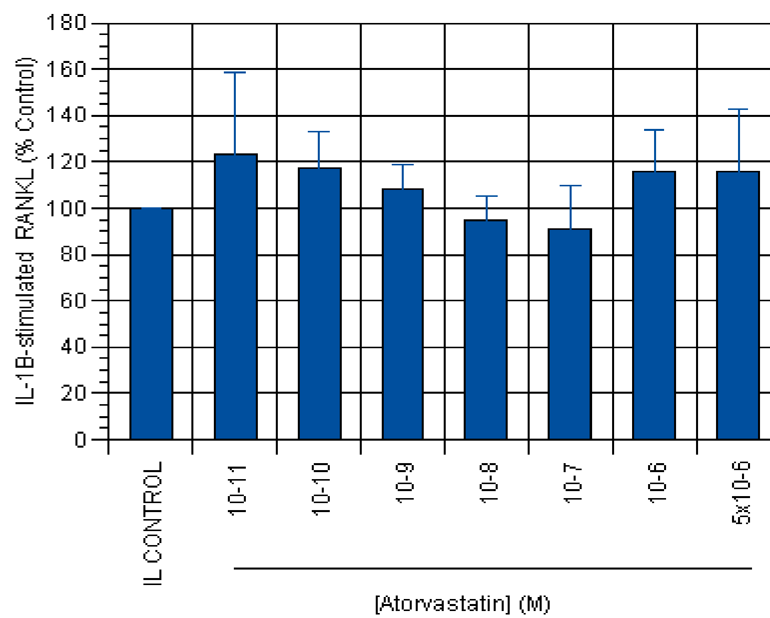

Figure 11. Effect of atorvastatin on IL-1β-stimulated RANKL production.

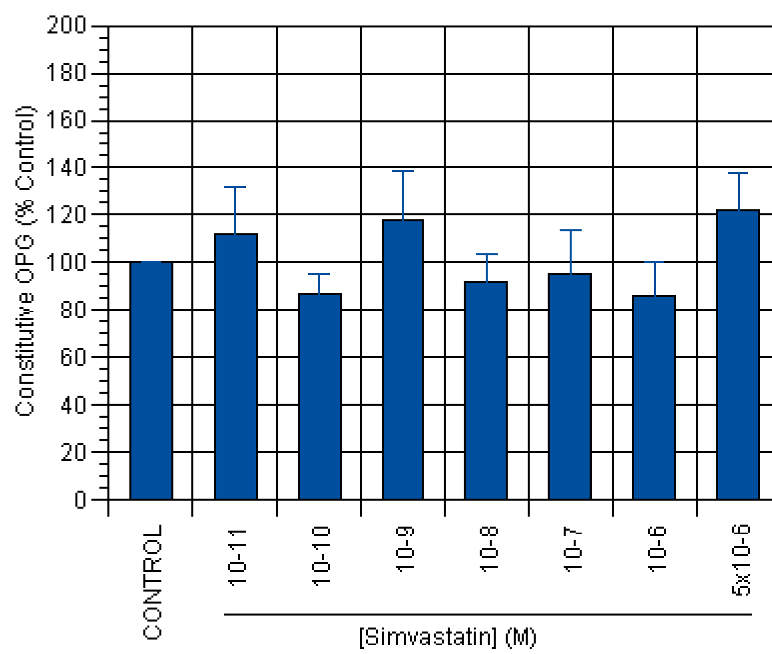

Figure 12. Effect of simvastatin on constitutive OPG production.

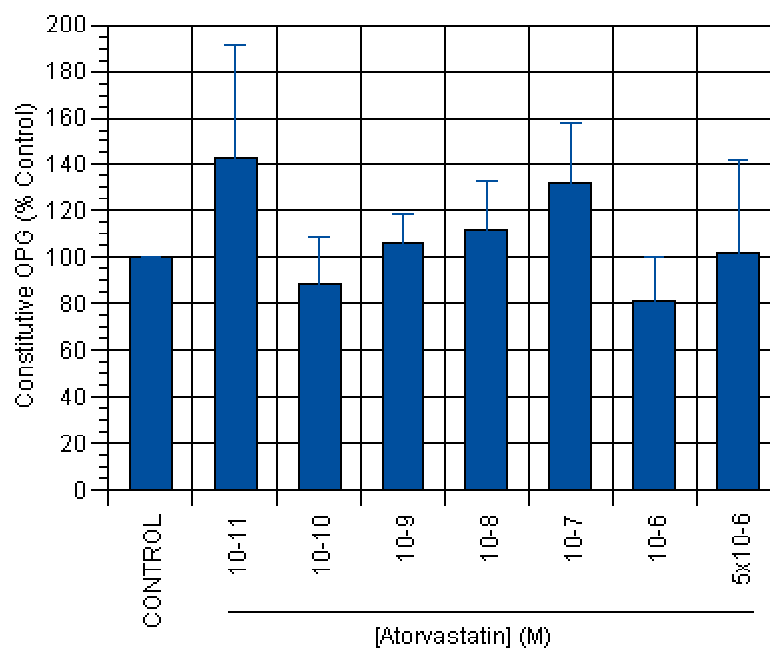

Figure 13. Effect of atorvastatin on constitutive OPG production.

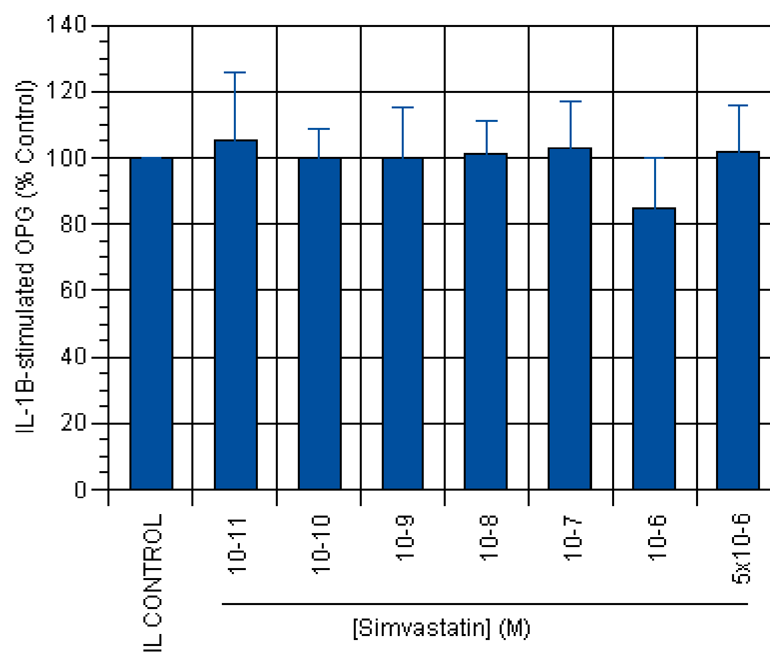

Figure 14. Effect of simvastatin on IL-1β-stimulated OPG production.

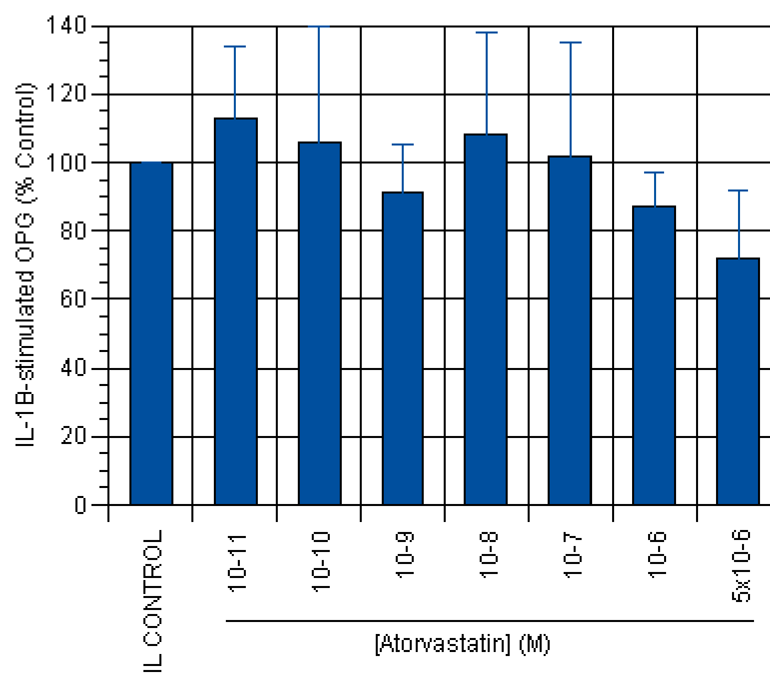

Figure 15. Effect of atorvastatin on IL-1β-stimulated OPG production.

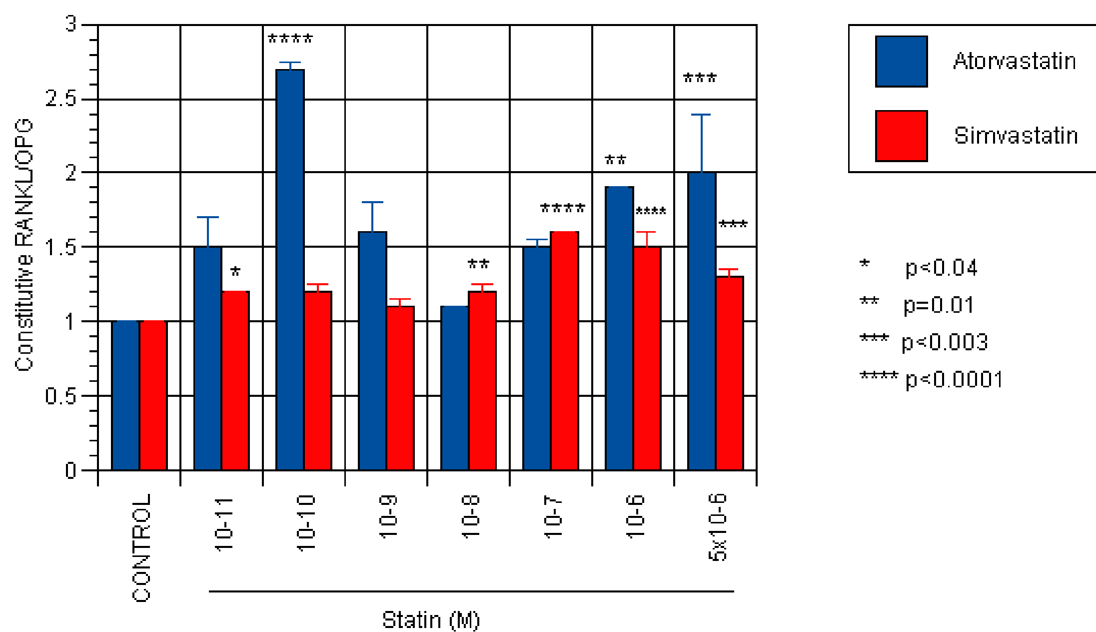

Figure 16. Effect of simvastatin or atorvastatin on constitutive RANKL/OPG ratios.

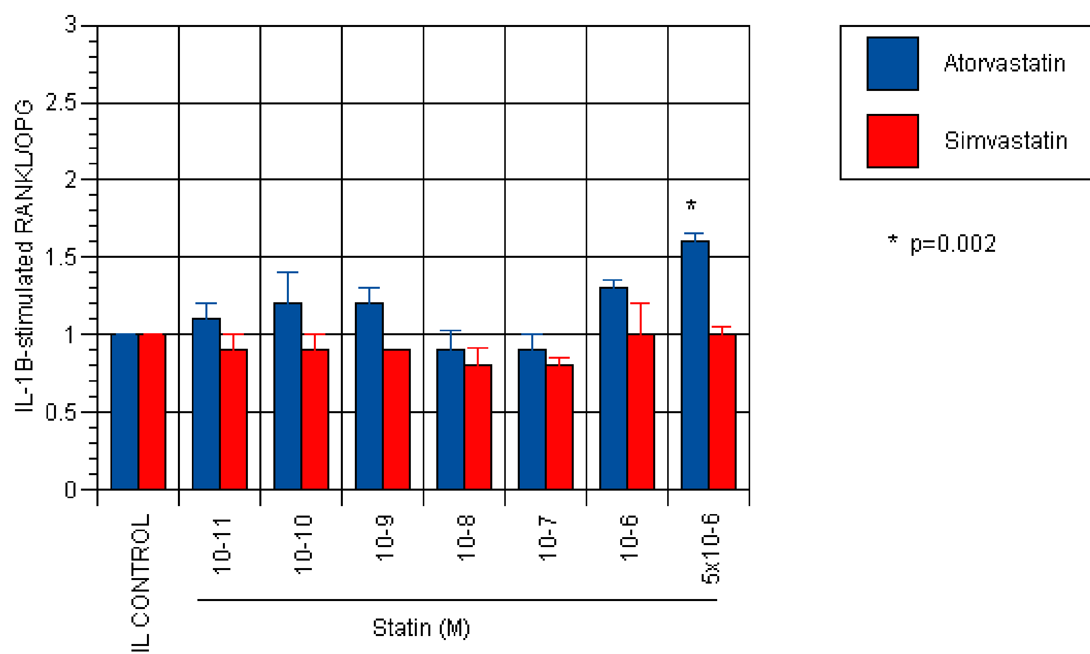

Figure 17. Effect of simvastatin or atorvastatin on IL-1β-stimulated RANKL/OPG ratios.

CHAPTER 4. DISCUSSION

This study examined the effect of simvastatin and atorvastatin on constitutive and IL-1β-stimulated production of OPG and RANKL by human gingival fibroblasts. Gingival fibroblasts, periodontal ligament fibroblasts, and osteoblasts are the three types of fibroblastic cells of mesenchymal origin in human periodontal tissue (47). Gingival fibroblasts make up 65% of the cell population in gingiva, and appear to be active participants in soft and hard tissue remodeling (51). As discussed in Chapter I, human gingival fibroblasts and periodontal ligament fibroblasts can produce RANKL and OPG. Therefore in addition to osteoblasts, it is now believed that fibroblasts are involved in the regulation of alveolar bone metabolism (47-50, 52). It is unknown however whether statins have an effect on OPG and RANKL production by human gingival fibroblasts. Therefore the purpose of this study was to evaluate the constitutive and IL-1β-stimulated production of OPG and RANKL by human gingival fibroblasts in the presence or absence of statins.

Simvastatin and atorvastatin were used in this study. They are two of the most frequently prescribed statins, either as monotherapy or in combination with other lipid lowering agents. Simvastatin is a synthetic derivate of a fermentation product of *Aspergillus terreus*. It is the only statin available in generic form. It also the most often used statin in research. Several investigators reported simvastatin-induced osteoblastic differentiation through its bone morphogenic protein-2 promoting effect, which resulted in greater new bone formation and improved fracture healing (53-57). Simvastatin has been used in the treatment of periodontal and peri-implant bone defects as well in socket preservation (58-60). One interesting fact about simvastatin metabolism is that the parent drug is without intrinsic activity. However, it is readily metabolized to simvastatin acid by nonenzymatic hydrolysis as well as by nonspecific esterases in the liver and other tissues. Simvastatin, but not simvastatin acid undergoes microsomal metabolism. Several metabolites resulting from microsomal oxidation are effective inhibitors of 3-hydroxy-3-methylglutaryl-CoA reductase and may contribute to the cholesterol lowering effect of simvastatin (61).

Atorvastatin is a completely synthetic product and has been found to be more potent compared to other statins at equal concentration and incubation time (28). Atorvastatin produces larger reductions of cholesterol and triglycerides compared with other drugs in this class (62). It has also been shown to reduce the levels of small, dense LDL (63). It is still awaiting its generic launch. It is metabolized solely by CYP3A4 (62). Liver metabolism produces two active hydroxyl metabolites, ortho-hydroxy-atorvastatin (o-OH-atorvastatin) and para-hydroxy-atorvastatin (p-OH-atorvastatin), and three corresponding inactive lactone metabolites (64). The active metabolites are equipotent to the parent drug in vitro. Elimination of the drug is mainly through the bile; renal excretion of radiolabelled drug and metabolites in urine is negligible (65). In contrast to other statins, dosage adjustment to avoid accumulation in patients with renal impairment is not necessary (66).

The effect of statins on OPG and RANKL production has been investigated in several cell culture systems and the results are controversial. Kaji et al. reported an increase in OPG and decrease in RANKL mRNA in mouse bone cell cultures when the cells were treated with mevastatin and simvastatin (67). The authors suggested that the modulation of OPG/RANKL ratio may be responsible for reduced osteoclast formation.

Similar results were reported by Viereck et al. when human osteoblasts were treated with atorvastatin (30). There was a time-dependent stimulatory effect of atorvastatin on OPG mRNA levels after 24 h and on OPG protein secretion after 48-72 h. The data suggested that atorvastatin enhanced osteoblastic differentiation and production of OPG, which may be contributory to the bone-sparing effects of statins. However, statins had an opposite effect on OPG production by endothelial and smooth muscle cells in vitro (68). The authors demonstrated that statins reduced TNF-α-induced OPG production in cultured human endothelial cells and smooth muscle cells. In addition, atorvastatin downregulated IL-1α-induced OPG production in endothelial cells. The effect of statins on TNF-α-induced OPG production was reversed by mevalonate and geranyl–geranyl pyrophosphate at the level of protein production and at the level of mRNA expression, suggesting that it was brought about by inhibition of the mevalonic acid pathway and protein prenylation.

Nellemann et al. examined the effect of simvastatin on OPG and adhesion molecules in type 2 diabetic patients with microalbuminuria (69). Since elevated OPG levels are associated with subclinical atherosclerosis in both type 1 and type 2 diabetes, these findings may reflect a simvastatin-calcification reduction (70, 71). Thus the effects of statins on RANKL and OPG production appear to be cell-culture specific.

In the present study, the concentrations of simvastatin and atorvastatin ranged from 5×10^{-6} to 1×10^{-11} M. This range is inclusive of the range of concentrations of these and other statins studied by others (25, 72, 73). Neither drug at any concentration tested, at any time point, was toxic to the fibroblasts (Figure 5). These findings confirm previously reported lack of toxicity of statins in concentration less than 5×10^{-6} M (28, 74). The lack of toxicity of IL-1β in the concentration tested (1×10^{-10} M) was based on results from previous studies in this lab (75). Evaluation of the effect of IL-1β on constitutive OPG production showed statistically significant difference on day 1 between control and 1×10^{-8} M, $p<0.0001$ (Figure 6). It continued on day 3 for the same concentration ($p<0.005$). On day 6, there was statistically significant difference for two concentrations of IL-1β (1×10^{-8} M and 1×10^{-10} M, $p=0.001$ (Figure 6). Thus under IL-1β stimulation, HGFs increase their OPG production, acting to prevent osteoclast formation and bone resorption. Similar findings were reported by Nagasawa et al. when human gingival fibroblasts were stimulated by LPS (48). The culture supernatant of LPS-stimulated gingival fibroblasts significantly reduced the number of TNF-receptor associated protein (TRAP) positive cells generated by culturing monocytes with RANKL and macrophage colony-stimulating factor (M-CSF). The results suggested that LPS-stimulated HGFs inhibit monocyte differentiation into osteoclasts through the production of OPG. Similarly Sakata et al. reported increased OPG level when human periodontal ligament cells under IL-1β stimulation (76). These effects of IL-1β on OPG production

are applicable to cell lines not present in the periodontium. When IL-1β was used as stimulus in chondrocyte cell culture, OPG and RANKL expression increased with IL-1β whereas M-CSF expression decreased. The results suggested that IL-1β suppresses the formation of osteoclast-like cells via increased OPG production and decreased M-CSF production in chondrocytes, thus decreasing cartilage turnover (77). In this study, a trend towards increasing RANKL production with IL-1β stimulation was seen, especially at days 3 and 6. No statistical significance was detected. Similar results were reported by Nukaga et al. when evaluating the effect of IL-1β on PDL cells (78). The authors concluded that IL-1β stimulated the expression of RANKL at messenger RNA (mRNA) and protein levels and the effect was partially mediated by endogenous PGE_2.

The effects of both drugs on constitutive or IL-1β-stimulated production of OPG and RANKL did not reach statistical significance at any concentrations. Both drugs increased the constitutive RANKL/OPG ratio, an effect favoring bone resorption. Statistical significance was detected for several of the concentrations tested. Simvastatin decreased slightly IL-1β-stimulated RANKL/OPG ratio (except for the highest concentrations of 5×10^{-6} M and 1×10^{-6} M). Atorvastatin increased IL-1β-stimulated RANKL/OPG ratio (except for 1×10^{-7} M and 1×10^{-8} M). Only the highest concentration of atorvastatin (5×10^{-6} M) produced statistically significant effect.

Thus the statins tested in this study differ minimally in their effect on RANKL/OPG ratios. The constitutive increase of RANKL/OPG ratio by atorvastatin and simvastatin could be a clinical reflection of the findings reported by Saxlin et al. (34). The study investigated the association between statin medication (simvastatin, atorvastatin or other) and periodontal infection in an adult non-diabetic, non-rheumatic population. The main outcome variable was the presence of periodontal infection measured in two ways: number of teeth with deepened periodontal pockets (4 mm or more) and number of teeth with deep periodontal pockets (6 mm or more). Bleeding on probing was also used as an outcome variable. Statin medication was weakly negatively associated with the presence and extent of periodontal infection. In stratified analyses, this beneficial effect was seen only in subjects with dental plaque or gingival bleeding. Among subjects with no gingival bleeding and to a lesser extent among subjects with no plaque, statin medication was associated with an increased likelihood of having teeth with deepened periodontal pockets. The results of the study suggested that statins have an effect on the periodontium that is dependent on the inflammatory condition of the periodontium.

Since the absence of bleeding on probing has a high negative predictive value for disease progression and deepened periodontal pockets are often associated with bone loss, the results reported by Saxlin et al. may be correlated to the increase in RANKL/OPG ratio by atorvastatin and simvastatin in the absence of IL-1β stimulation, a surrogate of inflammation (79). The finding that both statins increased constitutive RANKL/OPG ratios, suggests that in non-diseased state statins may influence the production of RANKL and OPG by HGFs to favor bone catabolism and confirms the suggestion by Saxlin et al. that the effect of statin medication may be dependent on the inflammatory condition of the periodontium.

The results from this study suggest that in non-stimulated cells statins may have a negative effect on RANKL/OPG balance by increasing the ratio and favoring bone resorption. Outcomes from other investigations are more favorable in terms of positive effect of statins on periodontal health. Therefore statins should not be dismissed as a potential therapeutic agent to prevent or recover bone loss in periodontal disease. Lindy et al. reported that statin use was associated with fewer numbers of pathologically deepened periodontal pockets compared with subjects not taking statin medication among chronic periodontitis patients (80). Pradeep et al. investigated the effectiveness of 1.2 mg simvastatin in a biodegradable controlled-release gel as an adjunct to scaling and root planing (SRP) in the treatment of chronic periodontitis (81). Clinical parameters were recorded at baseline before SRP and 1, 2, 4 and 6 months; they included modified sulcus bleeding index (mSBI), probing pocket depth (PPD), and relative attachment level (RAL). At the baseline and after 6 months, radiological assessment of intrabony defect (IBD) fill was done using computer-aided software. There was greater reduction in gingival index and PPD, and more RAL gain with significant IBD fill at sites treated with SRP plus locally delivered simvastatin in chronic periodontitis patients.

The results of this study are only applicable to the gingival fibroblast cell line used and should not be used to draw general conclusions about the gingival fibroblast population as an entity present in the gingiva. It should be emphasized, that the results of the study support the conclusion from previous investigators regarding the osteoprotective properties of gingival fibroblasts based on their elevated constitutive production of OPG (ng range) vs. RANKL (pg range) and confirm the role of gingival fibroblasts in bone metabolism. Under IL-1β stimulation, OPG production was increased by as much as 10-fold compared to control. Clinically, under inflammatory conditions such as periodontitis-induced bone damage, the increased production of OPG by gingival fibroblasts may act to abate bone resorption by decreasing the RANKL/OPG ratio. Further investigations are needed to verify the validity of our findings and determine the effects of statins on OPG and RANKL production by human gingival fibroblasts in the presence or absence of inflammatory mediators such as IL-1β.

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