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# Role of Prostaglandin Pathway and Alendronate-Based Carriers To Enhance Statin-Induced Bone

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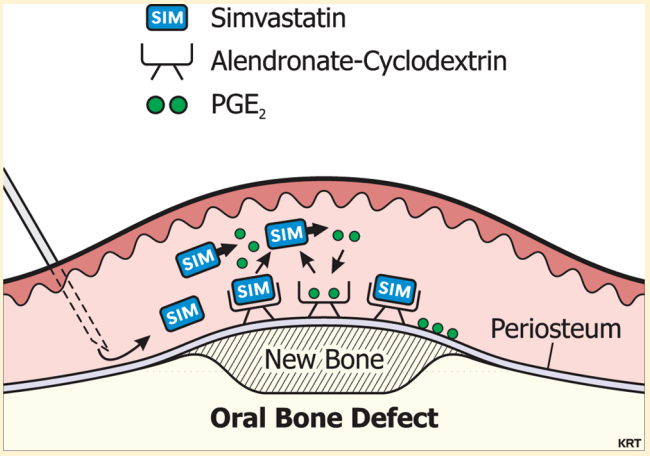
**ABSTRACT:** This study investigated the role of the prostaglandin (PG) pathway in locally applied, simvastatin-induced oral bone growth. The possibility of enhancing long-term bone augmentation with an alendronate-based carrier was initiated. Mandibles of 44 mature female rats were treated bilaterally with the following combinations: 2 mg of simvastatin in ethanol (SIM−EtOH), EtOH, 2 mg of simvastatin acid complexed with alendronate−beta-cyclodextrin conjugate (SIM/ALN−CD), ALN−CD, or ALN. Bone wash technology (injection of PBS and re-collection by suction) was used to sample injection sites at baseline (day 0), and 3, 7, 14, and 21 days post-treatment. After 21−24 or 48 days, histomorphometric analysis was done. The amount of $PGE_2$ in bone wash fluid was measured by ELISA, normalized by total protein, and compared between high and low bone growth groups (ANOVA) and correlated with subsequent bone histology at 21 days (Spearman). SIM-stimulated $PGE_2$ synthase and EP4 receptor mRNA in murine osteoblast and fibroblast cell lines were evaluated with real-time PCR. Single injections of 2 mg of SIM−EtOH induced significantly more new bone than control side after 21 days. $PGE_2$/protein ratios peaked at day 7 and were correlated with the subsequent 21-day new bone width. The correlations at day 14 between $PGE_2$ and new bone width changed to a negative relationship in the test group. SIM-stimulated osteoblasts expressed increased mRNA levels of PGE receptor EP4, while SIM activated PGE synthesis in fibroblasts. SIM/ALN−CD tended to preserve bone long-term. Findings suggest that PGE pathway activation and higher levels of $PGE_2$ during the first week following SIM-induced bone growth are desirable, and alendronate−beta-cyclodextrin conjugates not only act as tissue-specific carriers, but preserve new bone.




**KEYWORDS:** $PGE_2$, simvastatin, alendronate, cyclodextrin, bone growth

## INTRODUCTION

Oral and maxillofacial tumors, trauma, and extraction of teeth may result in severe loss of alveolar and mandibular bone, and there is increasing need for regeneration of bone for esthetics and dental implant therapy. Using exogenous grafts and growth factors (including bone morphogenetic protein [BMP]-2), trials to augment and induce local bone formation in the oral and maxillofacial area have been successful.[1] Primary examples would be adding bone for sinus augmentation or to increase deficient alveolar ridge width (Figure 1). Alveolar ridge defects are currently treated by surgically reflecting a flap of soft tissue (gingiva and mucosa) from the underlying bone, and then placing a bone graft (block or particles) onto the defect. The graft may be supplemented with growth factors such as BMP-2. The surgical flap is then replaced over the graft (perhaps with a membrane), sutured and allowed to heal. Significant cost and side-effects have been associated with this surgical approach. Less complicated, more reliable and predictable materials and therapeutic strategies are needed. The use of local bone anabolic injections could provide a cost-effective and patient-friendly approach to produce new bone in bone defects of the mouth and skull.

Locally applied hydrophobic statins (specific inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A [HMG-CoA] reductase in the production of cholesterol) were discovered to be potent stimulators of bone formation.[2] In a recent study, locally applied statin preserved greater residual alveolar ridge 2 weeks after treatment of extraction sockets and increased the bone mineral density after 4 weeks compared to the control group.[3] Stimulation of

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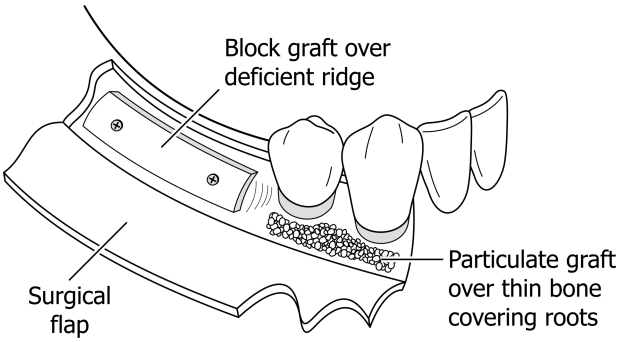


**Figure 1.** Graphic of human mandibular defects which are currently treated by osseous block grafts (e.g., deficient, narrow alveolar ridge) prior to dental implants, or particulate osseous grafts (e.g., over thin bone covering tooth roots). Both require surgical flap reflection with attendant morbidity and expense.

endogenous BMP-2 has long been claimed as the primary mechanism by which statins stimulate osteogenesis.[2]

Stein et al.[4] and Thylin et al.[5] showed that locally injected simvastatin induced new bone formation in rodent models in vivo. They demonstrated that locally applied simvastatin increased tissue swelling in a dose dependent manner, and the swelling was reduced by lowering the dose without affecting the bone formation. However, further reduction of inflammation with the cyclooxygenase (COX)- and prostaglandin (PG)-inhibiting nonsteroidal anti-inflammatory drug indomethacin nearly eliminated new bone growth,[4] suggesting that simvastatin-induced bone growth involved the prostaglandin pathway.

From a mechanism perspective, Maeda et al.[6] demonstrated that simvastatin increased the expression of BMP-2 and vascular endothelial growth factor (VEGF) in vitro. VEGF, BMP-2 and Cbfal also were expressed one day earlier in statin-treated sides than the other side treated with collagen alone in vivo.[7] Bradley et al.[8] found that a cyclooxygenase-2 inhibitor reduced both bone formation and BMP-2 production caused by simvastatin in a rat mandible model, which suggests cross-talk between BMP-2 and COX-2/PGE pathways in statin-induced bone formation. Obtaining direct evidence of the role of COX-2/PGE is clinically relevant since the most common pain medications prescribed following dental bone augmentation procedures contain COX inhibitors (e.g., aspirin, ibuprofen).

From a clinical perspective, simvastatin has been dissolved in an ethanol carrier to allow injection through small gauge needles, necessary within firm tissue overlying oral bone (e.g., gingiva and keratinized mucosa).[9,10] However, use of ethanol may be problematic due to pain at the injection site and dispersion of simvastatin−ethanol carrier into unwanted areas beyond the site of injection.[10] It has also been found that simvastatin-induced bone growth (new bone area and new bone width) after 24 days was resorbed over time, and only 23−55% of the new bone remained after 90 days.[11] Attenuation of this later resorption is critical in translation of local statin injections into clinically relevant dental bone augmentation.

To address these issues encountered in the clinical development of injectable statin therapy, the following experiments were undertaken. To provide direct evidence of the role of $PGE_2$ in simvastatin-induced bone augmentation, we analyzed in vivo $PGE_2$ production following simvastatin injections (using bone wash methodology) and then correlated these values to subsequent bone histology in a rat model. In addition, in vitro studies of osteoblast and fibroblast cell lines were used to dissect how simvastatin may activate $PGE_2$-associated genes in cells from the periosteal microenvironment. Finally, the possibility of using the molecular complex of simvastatin acid/alendronate−beta-cyclodextrin conjugates to deliver simvastatin acid and to preserve new bone formation in the jaw was explored.

## EXPERIMENTAL SECTION

**Preparation of the Molecular Complex of Simvastatin Acid/Alendronate−Beta-Cyclodextrin Conjugates.** Methods modified from Yoshinari et al.[12] were used to prepare the simvastatin acid/beta-cyclodextrin complexes. To obtain simvastatin acid, sodium hydroxide (0.1 N, 9 mL) was added to 6 mL of a SIM solution in ethanol (42 g/L). The resulting solution was stirred at 50 °C for 2 h to open up the lactone ring of SIM. Residual ethanol was removed under vacuum. The resulting solution was neutralized to pH 6.0 with HCl (0.1 M) and then was freeze-dried. The resulting dry powder was dispersed in acetone and centrifuged to remove NaCl. Simvastatin acid was then isolated from the supernatant. To prepare the molecular complex between simvastatin acid and alendronate−beta-cyclodextrin (ALN−CD[13]), simvastatin acid and ALN−CD were mixed in deionized water at the final concentrations of simvastatin acid 10 mg/mL and ALN−CD 75 mg/mL. The complex (pH 6.0) was stirred at room temperature for 1 h and then sterilized by filtration (0.22 $\mu$m) for the animal study.

**Animal Procedures.** All animal procedures were approved by the Institutional Animal Care & Use Committee at the University of Nebraska in accordance with National Institutes of Health guidelines. A bilateral mandible model[11] using 44 mature female Sprague−Dawley rats (Harlan Teklad, Madison, WI, USA) was used for these experiments. Prior to injections, all rats were sedated and maintained by isoflurane inhalation anesthesia.

Active drug treatments were randomized to right and left sides, and injections were placed over the lateral aspect of the mandible near the inferior angle. A 25 gauge 1 cm long needle was inserted 6 mm intramuscularly at the angle of the mandible, and the solution was deposited supraperiosteally.

*Short-Term Experiments.* 2 mg of simvastatin dissolved in 50 $\mu$L of 70% ethanol was injected on one side of the mandible (SIM−EtOH), and 50 $\mu$L of 70% ethanol alone was injected on the other (EtOH control) ($n$ = 20), as described previously.[11] A pilot study showed that a 2 mg simvastatin dose produced significant bone growth after 3 weeks without systemic complications. After healing (21 days) was attained, the animals were euthanized by $CO_2$ asphyxiation. Additional rats ($n$ = 12) were injected with alendronate−beta-cyclodextrin conjugates (ALN−CD) or alendronate in water (ALN) on one side of the mandible, versus water alone on the contralateral mandible (control), for additional bone wash studies.

*Long-Term Experiments.* To evaluate the ability of SIM/ALN−CD to create and preserve new bone compared to SIM−EtOH, right and left sides of the mandible were randomly injected with these two drug preparations as described above, and then histologic new bone was evaluated following euthanization at 48 days ($n$ = 12).

**Bone Wash Sampling.** Bone wash sampling was accomplished using the device modified from previous reports,[14] consisting of a 20 gauge outer cannula containing a 27 gauge inner cannula attached to a syringe. The outer cannula penetrated the skin and

muscle until it reached the bone surface at the site of experimental drug/control injections, where it formed a seal against the bone. 500 $\mu$L of PBS was injected onto the bone surface through the inner cannula, and the fluid containing bone wash then was collected using suction force through the outer cannula into sterile vials. Samples were obtained at baseline, day 7 and/or day 21 in one set of rats (SIM−EtOH and EtOH, ALN−CD and $H_2O$, ALN and $H_2O$; $n = 22$), and at day 3 and day 14 in the other set (SIM−EtOH and EtOH; $n = 10$) to provide at least a 7-day interval between sampling to minimize the impact of sampling trauma on subsequent samples.

**Histomorphometric Analysis of Decalcified Specimens.** To determine amount of bone growth and surrounding inflammation, samples were prepared for decalcified sections that included both sides of the mandible and overlying soft tissue.[4] The specimens were decalcified in 5% formic acid and embedded in paraffin, and subsequently 5 $\mu$m thick sections were cut at 200 $\mu$m intervals to give in-depth evaluation of the mandibular bone surface, and stained with hematoxylin-eosin.

Three sections for each animal (both sides in the same section) were analyzed without knowledge of group using a light microscope and a digital camera interfaced with Sigma Scan Pro 5 software (SPSS Inc., Chicago, IL, USA). As described previously,[11] mandibular width of old and new bone (micrographs previously shown in refs 4 and 11) was calculated as the average of three levels measured 1 mm apart beginning at the base of the mandible, and mandibular area of old and new bone was measured. Percent osteoblast surface (forming surface), flat-lining cell surface and osteoclast surface (resorbing surface) on the bone periphery were measured under 20× power after confirmation of cell type under 400× . Morphometric analysis of cell types in adjacent soft tissue was accomplished by grid intersection point counting at 400× power, yielding the percent of plasma cells, lymphocytes, neutrophils, macrophages, monocytes, muscle, fibroblasts, collagen and blood vessels.

**Analysis of Biomarkers.** *Total Protein.* The amount of total protein in bone wash samples was quantified using a BCA protein assay kit (Pierce Chemical, Rockford, IL, USA). Twenty five microliters of each standard or unknown sample were used, and the working range was 20−2000 $\mu$g/mL. The absorbance was measured at 562 nm using a microplate reader (BioTek, North Seattle, WA, USA), and the concentration of total protein in each bone wash sample was calculated.

*Prostaglandin $E_2$.* A Luminex Prostaglandin $E_2$ kit (Cayman Chemical, Ann Arbor, MI, USA) was used to quantify the amount of $PGE_2$ in samples. This assay is based on the competition between $PGE_2$ of unknown concentration in sample and phycoerytherin-conjugated $PGE_2$ tracer of known concentration for a limited amount of $PGE_2$ monoclonal antibody binding site on the microsphere beads. In brief, 100 $\mu$L of standard or sample was placed into appropriated wells into which phycoerytherin-conjugated $PGE_2$ tracer and beads coupled with monoclonal antibody for $PGE_2$ were added. Raw data were reported as fluorescence intensity (FI) using the Bio-Plex reader. The standard curve and the concentration of samples were calculated using data analysis tools provided by Cayman Chemical (sensitivity = 35 pg/mL).

**Cell Culture.** In order to explore potential mechanisms of $PGE_2$ production, prominent cells of the periosteal microenvironment (osteoblasts and fibroblasts) stimulated by simvastatin were evaluated for activation of genes important to the $PGE_2$ bone anabolic pathway. Murine calvarial preosteoblasts MC3T3-E1 subclone 14 (CRL-2594, Manassas, VA, USA) and murine embryonic fibroblasts (CF1, ATCC # SCRC-1040) were cultured in α-MEM (Hyclone, Logan, UT) with 1 mM sodium pyruvate containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 $\mu$g/mL streptomycin and 0.5 $\mu$g/mL amphotericin B (Invitrogen, Carlsbad, CA, USA) under a humidified 5% $CO_2$ atmosphere. Cells were subcultured and seeded in 6 well cell culture plates at a concentration of 15,000 cells/cm$^2$. When cells reached approximately 80% confluence, the concentration of FBS was decreased to 2% in medium before the treatment.

**Real-Time (Semiquantitative) PCR.** The cells were incubated with and without $10^{-7}$ M simvastatin dissolved in ethanol carrier. The concentration of ethanol was 0.01% in medium. Total RNA was extracted 6−72 h after treatment using TRIzol (Invitrogen) and isolated according to the manufacturer's protocol. An RNA pellet was dissolved in diethylpyrocarbonate, treated with (DEPC) $H_2O$ and stored at −70 °C. cDNA was synthesized using the Superscrip III first-strand synthesis system (Invitrogen, Carlsbad, CA, USA) by manufacturer's instruction. The real time PCR was performed in triplicate using Real master mix SYBR Rox (Fisher Scientific, Pittsburgh, PA, USA). One microliter of cDNA was amplified in a 20 $\mu$L master mix containing hot master Tag DNA polymerase, 10 mM magnesium acetate, 1.0 mM dNTPs with dUTP and SYBR fluorescent dye solution. The oligonucleotide primers were designed by Plexor primer design software (Promega, Madison, WI, USA), and the primers used for microsomal $PGE_2$ synthase and EP4 had the following sequences: m$PGE_2$ synthase, forward, 5′-ACTTCATCTCCTC-CGTCCTGG-3′; reverse, 5′-AAGGAGGTGACCGAGTTTT-GC-3′; EP4, forward, 5′-CCTTCATCCTACTTTTCTTCGG-TCTG-3′; reverse, 5′-TGTAGAAGTAGGCGTGGTTGATGG-3′ (UNMC Eppley Molecular Biology Core Laboratory, Omaha, NE, USA). $\beta$-Actin was used for the housekeeping gene. Each primer was used at a 300 nM concentration. Amplication protocol was as follows: initial temperature denaturation at 94−95 °C for 1 min, 40 cycles of 94 °C for 20 s for cycled temperature denaturation; 55 °C for 20 s for annealing; and 68 °C for 30 s for extension. Dissociation curve was obtained after last cycle to validate the specificity of the primer. The comparative ΔΔCt method was used to assess relative changes in mRNA levels among samples.

**Statistical Analyses.** Histological measurements within animals (between injection groups) were compared using analysis of variance (ANOVA). The concentration of $PGE_2$ was normalized by total protein in each sample. $PGE_2$ levels among bone wash sampling times (baseline, 3, 7, 14, and 21 days), between injection groups and between animals with high eventual bone growth (area of new bone >0.5 mm$^2$) and low eventual bone growth (area of new bone <0.5 mm$^2$) were compared using repeated measures ANOVA. Spearman correlation coefficients were used to compare earlier biomarker levels (conventional and rank-transformed data[15]) with subsequent bone histology at 21 days. mRNA levels were compared using ANOVA and Bonferroni post hoc tests.

## RESULTS

Single injections of 2 mg of simvastatin in ethanol on one side of the mandible produced significantly more new bone width and new bone area than contralateral ethanol alone after 21 days (Figure 2). At that point in time, the ratio of bone forming to bone resorbing surface was 3.65:1 around simvastatin injections

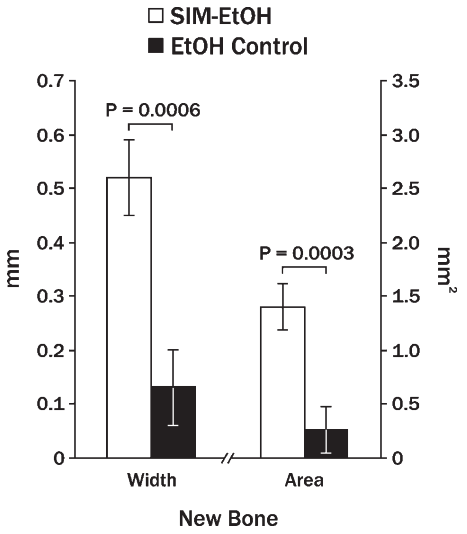


**Figure 2.** Mean new mandibular bone width and area (±SEM) 21 days following a single injection of 2 mg of simvastatin in 70% ethanol (SIM−EtOH) compared to contralateral ethanol alone (EtOH).

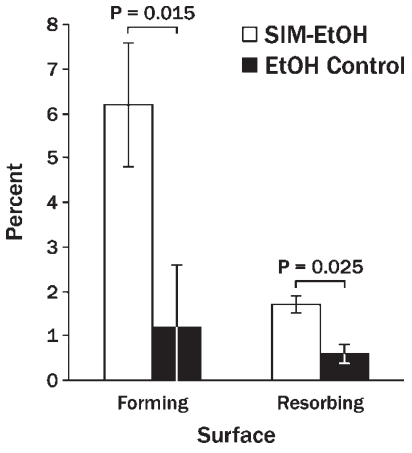


**Figure 3.** Mean percent (±SEM) of bone forming and bone resorbing surface 21 days following a single injection of 2 mg of simvastatin in ethanol (SIM−EtOH) compared to contralateral ethanol alone (EtOH).

and 2.0:1 around control injections (Figure 3). The area adjacent to the bone (displacing muscle tissue) was composed primarily of fibroblast-like cells and collagen (Figure 4), with significantly higher values on the simvastatin side.

$PGE_2$ was detectable in all bone wash samples. $PGE_2$/protein ratios peaked at day 7 in both simvastatin (2822.5 ± 673.2) and control (246.7 ± 673.2) sites, but were not statistically different between simvastatin and control groups. However, when sites that showed higher levels of bone growth (>0.5 $mm^2$ new bone area) at 21 days postinjection were compared to sites that showed limited bone growth (<0.5 $mm^2$ new bone area), $PGE_2$/protein was significantly higher in the high growth sites after 7 days (Figure 5). Day 7 levels of $PGE_2$/protein trended toward a positive correlation (Spearman) with the amount of 21-day new bone width (Table 1).

All other significant correlations between early SIM-stimulated $PGE_2$ and subsequent histology are outlined in Table 1.

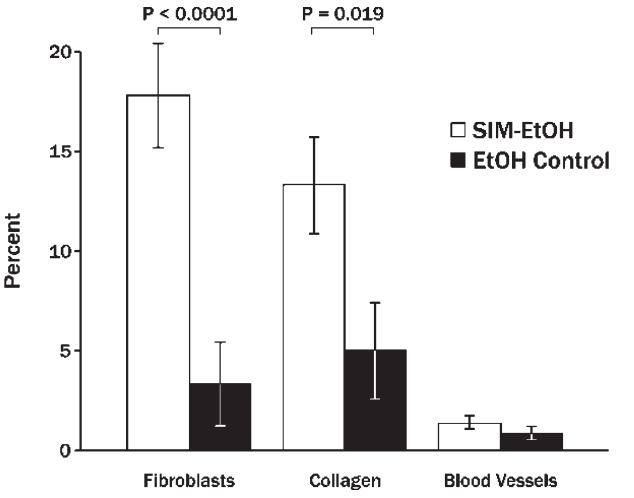


**Figure 4.** Mean percent (±SEM) of area occupied by fibroblast-like cells, collagen-like tissue and blood vessels displacing muscle adjacent to the injection site 21 days following 2 mg simvastatin/ethanol injections (SIM−EtOH) compared to contralateral ethanol alone (EtOH).

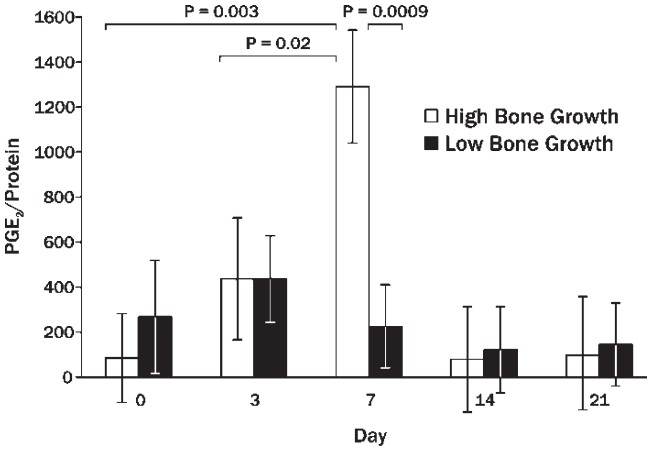


**Figure 5.** Mean $PGE_2$/protein ratio (±SEM) at various days postinjection preceding 21-day determination of high amounts of mandibular new bone area (>0.5 $mm^2$; high bone growth) compared to limited new bone area (<0.5 $mm^2$; low bone growth).

**Table 1. Significant Spearman Correlations between Simvastatin-Stimulated Early $PGE_2$ Levels and Subsequent Bone Histology**

| day | side | histology measure | *R* value/*p* value |
|---|---|---|---|
| 0 | SIM−EtOH | % blood vessel | −0.94/0.005 |
| 7 | SIM−EtOH | new bone width | 0.58/0.07 |
| | SIM−EtOH | % resorb. surface | 0.75/0.01 |
| | SIM−EtOH | % collagen | 0.68/0.03 |
| | SIM−EtOH | % blood vessel | 0.73/0.02 |
| 14 | SIM−EtOH | new bone width | −0.74/0.01 |

The percentage of blood vessels around bone was negatively correlated with $PGE_2$/protein in the simvastatin specimens at baseline. This changed to a significant positive correlation for $PGE_2$/protein at day 7. Finally, the correlation between day 14

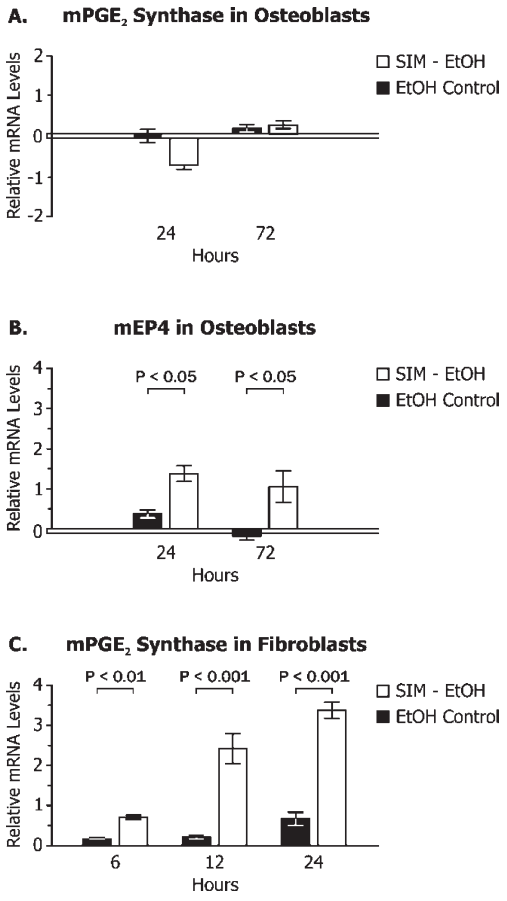


**Figure 6.** $PGE_2$ synthase (A) and EP4 (B) mRNA expression in rodent preosteoblasts, and $PGE_2$ synthase mRNA expression in rodent fibroblasts (C) in cell culture following stimulation with simvastatin/ethanol (SIM−EtOH) compared to ethanol alone (EtOH control) (mean relative change from zero at baseline ± standard deviation).

$PGE_2$/protein and day 21 new bone width changed to a negative relationship in the SIM group. Pearson correlation coefficients were similar to the Spearman correlations, but with generally higher $R$ values (data not shown). Furthermore, correlations using the rank-transformed bone wash data showed a similar pattern, strongly confirming the impact of $PGE_2$ at day 7 on day 21 new bone width ($r = 0.63$, $P = 0.003$), new bone area ($r = 0.59$, $P = 0.006$) and percent resorbing surface ($r = 0.58$, $P = 0.008$).

Data shown in Figure 5 and Table 1 suggest a close relationship between day 7 amounts of $PGE_2$ and subsequent bone growth. However, in the following week the $PGE_2$ levels had a negative impact on this future bone growth in the simvastatin group. Using linear regression analysis, 77% of new bone area variability was explained by $PGE_2$/protein ($R^2 = 0.769$, $P = 0.01$) on simvastatin sides. This association held for $PGE_2$/protein in the control specimens for new bone width ($R^2 = 0.920$, $P = 0.0001$) and new bone area ($R^2 = 0.730$, $P = 0.0046$).

When preosteoblasts were stimulated with simvastatin in culture, the relative amount of mRNA for $PGE_2$ synthase, the primary enzyme driving $PGE_2$ production, was not increased (Figure 6A). However, significant increase in the EP4 mRNA, the primary $PGE_2$ receptor in this cell line, was noted after 24 and 72 h (over 2-fold, Figure 6B). In contrast to the osteoblasts, a fibroblast cell line representing the main cellular component of the periosteal microenvironment showed an increase in the

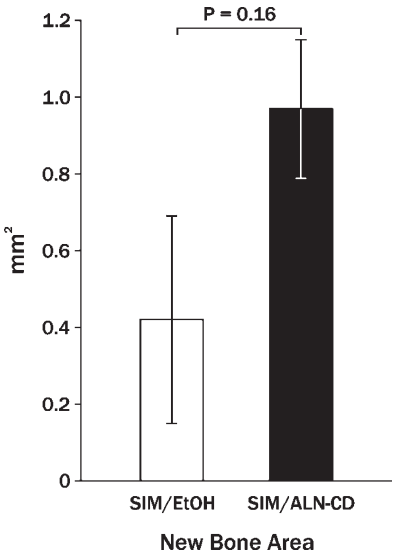


**Figure 7.** Mean new mandibular bone area (±SEM) 48 days following a single injection of 2 mg simvastatin in 70% ethanol (SIM−EtOH) compared to 2 mg of simvastatin in alendronate−cyclodextrin (SIM/ALN−CD).

mRNA for $PGE_2$ synthase after 6−24 h (5-fold over control at 24 h, Figure 6C).

The previous data indicate that injectable simvastatin is capable of stimulating new bone formation associated with $PGE_2$ pathway activation, but the new bone is susceptible to subsequent resorption and lack of localization to the site of injection.[11] Therefore, simvastatin acid (active form of simvastatin) was complexed with alendronate−beta-cyclodextrin conjugate (ALN−CD), an osteotropic drug carrier containing the antiresorption drug alendronate. Long-term (48 day) results of single injections of 2 mg of simvastatin/alendronate−beta-cyclodextrin conjugate on rat mandibles compared with 2 mg of simvastatin in ethanol (same preparation as the 21-day experiments) showed a trend toward more new bone area surviving in the SIM/ALN−CD group (Figure 7). At an earlier time period (24 days), the bone at the SIM/ALN−CD injection site had a lower percent osteoclast/mm of bone surface than SIM−EtOH ($0.3 \pm 0.2$ vs $1.0 \pm 0.2$%, $P = 0.007$) and number of osteoclasts/mm ($0.14 \pm 0.09$ vs $0.50 \pm 0.09$, $P = 0.009$). At the 48 day time, both SIM−EtOH and SIM/ALN−CD had minimal forming or resorbing bone surface (mostly quiescent bone) without elevated fibroblasts, collagen or signs of inflammation in surrounding soft tissue. Injection of the ALN−CD component of the complex did not stimulate any more $PGE_2$ production in bone washes after 7 days than water injections alone, although ALN in water caused a significant increase in 7 day $PGE_2$ (Figure 8).

## DISCUSSION

$PGE_2$ is a major product of the COX pathway and has been proposed as a mediator of both bone formation and resorption.[16−18] $PGE_2$ also appears to be associated with angiogenesis (Table 1). Statins are known to regulate COX-2 expression in different ways depending on the amount of local inflammation. For instance, statins stimulated cells of the monocyte lineage to express COX-2 gene and produce $PGE_2$ by controlling small G protein after the blockade of the mevalonate pathway.[19] However, LPS-stimulated expression of both COX-2 and $PGE_2$ was suppressed by

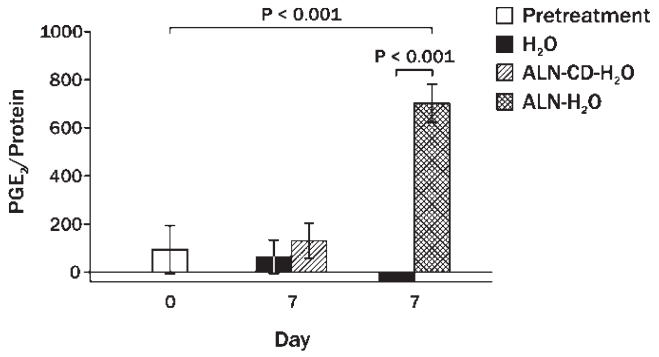


**Figure 8.** Mean $PGE_2$/protein ratio (±SEM) prior to and 7 days postinjection of alendronate (ALN) or alendronate−beta-cyclodextrin (ALN−CD) both diluted in water compared to water alone.

statins in a Rac and NF-kB-dependent manner.[20] Statin-induced $PGE_2$ production in the bone microenvironment was a unique aspect of the current study. This is the first investigation to our knowledge to follow $PGE_2$ levels chronologically after local simvastatin application, and relate this pattern to eventual bone growth. Seven days after SIM injections, the bone wash $PGE_2$ levels from the surrounding area were significantly correlated with subsequent new bone width. Sites with higher bone growth had significantly more $PGE_2$/protein than sites with low bone growth. In fact, 77% of the variability of new bone was explained by $PGE_2$/protein levels around simvastatin injections in the current study. $PGE_2$ levels also correlated with the percent of resorbing surfaces seen in the 21 day histology.

Limitations of the bone wash technique include (1) contamination of the sample with blood products; (2) slight variability in the amount of wash retrieved; and (3) sampling at a single point on the periosteum without intrabony access. Bone wash $PGE_2$ levels in the current study have been normalized with essentially albumin, which is not locally produced and helps standardize for differences in sample volume and bleeding contamination. Bone wash measurements used here are not intended to represent definitive values, but rather to rank low versus high $PGE_2$ amounts, then compare these to low versus high subsequent bone growth in the same area. Therefore, correlations also were analyzed using rank transformation of bone wash data, where values were ranked from low to high. Such data calculation avoided much of the potential variability around the continuous biomarker measurements. Results confirmed the correlations between day 7 $PGE_2$ and subsequent new bone width and percent resorbing surface.

Alternate sampling methods to assess bone $PGE_2$ levels would include obtaining bone and periosteal biopsies, with the disadvantages of very small sample amounts disrupting the experimental site and jeopardizing subsequent biochemical or histologic measurements. Unpublished pilot studies in our laboratory have shown that harvesting bone and periosteum post-mortem around SIM injection sites in rat palates resulted in $PGE_2$ levels undetectable by ELISA. Larger mandibular specimens containing portions of the overlying masseter muscle have been sufficient for biochemical analysis,[8] but these animals were euthanized. Gingival crevicular fluid (GCF) samples are another option in humans though not rats, but a previous human study showed that GCF and bone wash measurements of bone turnover markers were significantly different, presumably because GCF is mostly produced further from the bone surface.[14] However, multiple bone wash measurements of untreated areas over an 8−20 day period were not different, supporting the reproducibility of the method.

The possible sources of $PGE_2$ production and its interaction with bone forming cells remain a matter of speculation. However, increased in vivo osteoblasts and bone-forming surface were noted in the simvastatin injection sites (Figure 3). In addition, fibroblasts and collagen were enhanced adjacent to the bone forming surfaces as a result of SIM injections (Figure 4). Our in vitro studies confirmed that rodent fibroblast cell lines stimulated with SIM, but not preosteoblast cells, showed elevated activation of $PGE_2$ synthase within the first 24 h. According to our other experiments, expression of $mPGE_2$ synthase and release of $PGE_2$ in osteoblast and fibroblast cell culture were correlated (data not shown). Furthermore, EP4, the primary $PGE_2$ receptor on the osteoblasts, was upregulated within 24 h. These results suggest that simvastatin stimulation of $PGE_2$ synthesis by fibroblasts and activation of $PGE_2$ receptor in osteoblasts may be one of the mechanisms of osteogenesis by local statin delivery. While systemic doses of statin have generally been shown to down-regulate $PGE_2$ production in rat tissues[21] and humans,[22] local application of statins and prostaglandin E receptor agonists (i.e., PGE upregulation) have shown promise in stimulating bone grown in animal models.[4,5,8,11,23] In vitro statin-induced stimulation of COX-2 gene transcription and $PGE_2$ formation have been demonstrated in other cell types, such as macrophages[19] and keratinocytes.[24] Taken together, these findings are consistent with locally applied simvastatin enhancing the PGE pathway among cells in the bone microenvironment.

Even though $PGE_2$ levels were considerably lower 14 days after SIM injections, $PGE_2$ values at that time point were negatively correlated with new bone width one week later. These findings suggest that a transition was occurring between day 7 and day 14 whereby the timing and local concentration of $PGE_2$ in the bone turnover cycle may be important to eventual bone augmentation. It has been found previously that PGE can have both anabolic and catabolic activity depending on its local concentration.[25] In a recent review,[26] it was concluded that $PGE_2$ contributed to catabolic or anabolic actions dependent on the factor inducing PG production and the local cellular milieu. For instance, $PGE_2$ stimulation of bone resorption in rodents is primarily dependent on $G\alpha_s$ activation in the EP4 receptor, particularly among inflammatory cells where proinflammatory cytokines IL-1, IL-6, IL-11, IL-17 and tumor necrosis factor-α drive continuous PG production and upregulate receptor activator of nuclear factor kB ligand (RANKL). On the other hand, bone formation is enhanced by intermittent higher dose pulses of PGE (potentially induced by intermittent simvastatin[11]) via EP2 or EP4 receptors on osteoblasts, and cAMP/PKA or EPAC/NFAT, CREB or AP-1 pathways. Our previous studies indicated that $PGE_2$ inhibition during the first week following simvastatin application caused reduced local bone formation and BMP-2 secretion.[4,8] Alternatively, reduction of $PGE_2$ at the 14 day time point may be important for improved odds of bone preservation.

The delivery system for simvastatin appears to be crucial to achieving clinically relevant bone growth in the oral/facial region. The standard carrier for simvastatin injections in the current rat bilateral mandible study was 70% ethanol, compared to earlier studies using a simvastatin suspension in methylcellulose gel.[11] The advantages of the ethanol carrier over methylcellulose include (1) less viscosity allowing use of small gauge needles for less trauma and leakage in dense tissue (e.g., masseter muscle

or gingival papilla), (2) solution versus suspension to allow better distribution of the drug, and (3) better dissipation of the carrier. However, clinical concerns with the ethanol carrier, such as potential pain at the site of injection, dispersion of the drugs away from the treatment site of injection, and need to preserve new bone over time, make evaluation of carrier alternatives important.

While SIM−EtOH caused significant new bone growth with efficient injections, increased resorbing bone surface at injection sites was noted after 21−24 days compared to EtOH alone (Figure 3), suggesting potential bone resorption in the future. The longer-term (48 days) evaluations indicated that only 30% of the early bone formation (21 days) stimulated by 2 mg of SIM−EtOH was retained (Figures 2 and 7). This is similar to the SIM−methylcellulose preparations after 90 days.[11] By contrast, the same dose of SIM in a ALN−CD delivery system averaged nearly 70% retention of early bone (Figures 2 and 7). This is consistent with the lower percent of osteoclasts/mm and number of osteoclasts/mm seen at SIM/ALN−CD versus SIM−EtOH sites at 21 days, suggesting the likelihood that reduced subsequent resorption (between days 21 and 48) would occur. In addition, the ALN−CD conjugate itself did not stimulate significant $PGE_2$ after 7 days in vivo, while ALN alone stimulated a highly significant increase in $PGE_2$ (Figure 8). Interestingly, we have reported that ALN−CD has an anabolic effect on rat mandibular bone, probably by sequestering endogenous $PGE_2$ from the bone microenvironment.[13] $PGE_2$ may complex readily with the cyclodextrin conjugate and form a reservoir for sustained release of $PGE_2$. In the current study, simvastatin acid occupied much of the CD cavities, thereby preventing the $PGE_2$ sequestration potential. The data also suggest that ALN conjugated to cyclodextrin could not stimulate the $PGE_2$ bone anabolic pathway during the first week, yet the anabolic effects of simvastatin acid that complexed with the cyclodextrin were intact. Probably, ALN−CD was gradually metabolized and alendronate or its derivative might be released locally, thereby inhibiting osteoclast activity and bone resorption. Additional effort has been undertaken in our laboratories to better understand the local metabolism of ALN−CD. In addition, longer term studies evaluating new bone in function (e.g., around an implant) using histomorphometry and bone stress tests should be performed.

We conclude that (1) simvastatin in ethanol carrier is a potent inducer of mandibular bone growth; (2) there is direct evidence for the importance of $PGE_2$ in the early bone anabolic effects of locally applied simvastatin; and (3) the use of ALN−CD carriers hold promise to enhance simvastatin bone augmentation procedures because they reduce potential side-effects (pain), may target/retain drug to the desirable bone surface, and preserve the newly formed bone.

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## REFERENCES

(1) Jung, R. E.; Windisch, S. I.; Eggenschwiler, A. M.; Thoma, D. S.; Weber, F. E.; Hämmerle, C. H. A randomized-controlled clinical trial evaluating clinical and radiological outcomes after 3 and 5 years of dental implants placed in bone regenerated by means of GBR techniques with or without addition of BMP-2. *Clin. Oral Implants Res.* **2009**, *20*, 660–666.

(2) Mundy, G.; Garrett, R.; Harris, S.; Chan, J.; Chen, D.; Rossini, G.; Boyce, B.; Zhao, M.; Gutierrez, G. Stimulation of bone formation in vitro and in rodents by statins. *Science* **1999**, *286*, 1946–1949.

(3) Wu, Z.; Liu, C.; Zang, G.; Sun, H. The effect of simvastatin on remodeling of the alveolar bone following tooth extraction. *Int. J. Oral Maxillofac. Surg.* **2008**, *37*, 170–176.

(4) Stein, D.; Lee, Y.; Schmid, M. J.; Killpack, B.; Genrich, M. A.; Narayana, N.; Marx, D. B.; Reinhardt, R. A. Local simvastatin effects on mandibular bone growth and inflammation. *J. Periodontol.* **2005**, *76*, 1861–1870.

(5) Thylin, M. R.; McConnell, J. C.; Schmid, M. J.; Reckling, R. R.; Ojha, J.; Bhattacharyya, I.; Marx, D. B.; Reinhardt, R. A. Effects of statin gels on murine calvarial bone. *J. Periodontol.* **2002**, *73*, 1141–1148.

(6) Maeda, T.; Kawane, T.; Horiuchi, N. Statins augment vascular endothelial growth factor expression in osteoblastic cells via inhibition of protein prenylation. *Endocrinology* **2003**, *144*, 681–692.

(7) Wong, R. W.; Rabie, A. B. Statin collagen grafts used to repair defects in the parietal bone of rabbits. *Br. J. Oral Maxillofac. Surg.* **2003**, *41*, 244–248.

(8) Bradley, J. D.; Cleverly, D. G.; Burns, A. M.; Helm, N. B.; Schmid, M. J.; Marx, D. B.; Cullen, D. M.; Reinhardt, R. A. Cyclooxygenase-2 inhibitor reduces simvastatin-induced bone morphogenetic protein-2 and bone formation in vivo. *J. Periodont. Res.* **2007**, *42*, 267–273.

(9) Morris, M. S.; Lee, Y.; Lavin, M. T.; Giannini, P. J.; Schmid, M. J.; Marx, D. B.; Reinhardt, R. A. Injectable simvastatin in periodontal defects and alveolar ridges: pilot studies. *J. Periodontol.* **2008**, *79*, 1465–1473.

(10) Rutledge, J.; Schieber, M. D.; Chamberlain, J. M.; Byarlay, M.; Killeen, A. C.; Giannini, P. J.; Marx, D. B.; Reinhardt, R. A. Simvastatin application to augment facial jaw bone in a dog model: pilot study. *J. Periodontol.* **2010**, Nov 2 [epub ahead of print].

(11) Lee, Y.; Schmid, M. J.; Marx, D. B.; Beatty, M. W.; Cullen, D. M.; Collins, M. E.; Reinhardt, R. A. The effect of local simvastatin delivery strategies on mandibular bone formation in vivo. *Biomaterials* **2008**, *29*, 1940–1949.

(12) Yoshinari, M.; Matsuzaka, K.; Hashimoto, S.; Ishihara, K.; Inoue, T.; Oda, Y.; Ide, T.; Tanaka, T. Controlled release of simvastatin acid using cyclodextrin inclusion system. *Dent. Mater. J.* **2007**, *26*, 451–456.

(13) Liu, X.-M.; Wiswall, A. T.; Rutledge, J. E.; Akhter, M. P.; Cullen, D. M.; Reinhardt, R. A.; Wang, D. Osteotropic $\beta$-cyclodextrin for local bone regeneration. *Biomaterials* **2008**, *29*, 1686–1692.

(14) Wilson, A. N.; Schmid, M. J.; Marx, D. B.; Reinhardt, R. A. Bone turnover markers in serum and periodontal microenvironments. *J. Periodont. Res.* **2003**, *38*, 355–361.

(15) Conover, W. J. *Practical Non-parametric Statistics*; Wiley: New York, 1971; p 337.

(16) Yoshida, K.; Oida, H.; Kobayashi, T.; Maruyama, T.; Tanaka, M.; Katayama, T.; Yamaguchi, K.; Segi, E.; Tsuboyama, T.; Matsushita, M.; Ito, K.; Ito, Y.; Sugimoto, Y.; Ushikubi, F.; Ohuchida, S.; Kondo, K.; Nakamura, T.; Narumiya, S. Stimulation of bone formation and prevention of bone loss by prostaglandin E EP4 receptor activation. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 4580–4585.

(17) Ramirez-Yañez, G. O.; Seymour, G. J.; Walsh, L. J.; Forwood, M. R.; Symons, A. L. Prostaglandin E2 enhances alveolar bone formation in the rat mandible. *Bone* **2004**, *35*, 1361–1368.

(18) Liu, X. H.; Kirschenbaum, A.; Yao, S.; Levine, A. C. Cross-talk between the interleukin-6 and prostaglandin E(2) signaling systems results in enhancement of osteoclastogenesis through effects on the osteoprotegerin/receptor activator of nuclear factor-{kappa} B (RANK) ligand/RANK system. *Endocrinology* **2005**, *146*, 1991–1998.

(19) Chen, J. C.; Huang, K. C.; Wingerd, B.; Wu, W. T.; Lin, W. W. HMG-CoA reductase inhibitors induce COX-2 gene expression in murine macrophages: role of MAPK cascades and promoter elements for CREB and C/EBP beta. *Exp. Cell. Res.* **2004**, *301*, 305–319.

(20) Habib, A.; Shamseddeen, I.; Nasrallah, M. S.; Antoun, T. A.; Nemer, G.; Bertoglio, J.; Badreddine, R.; Badr, K. F. Modulation of COX-2 expression by statins in human monocytic cells. *FASEB J.* **2007**, *21*, 1665–1674.

(21) Nassar, P. O.; Nassar, C. A.; Guimaraes, M. R.; Acquino, S. G.; Andia, D. C.; Muscara, M. N.; Spolidorio, D. M.; Rossa, C., Jr.; Spolidorio, L. C. Simvastatin therapy in cyclosporine A-induced alveolar bone loss in rats. *J. Periodontol. Res.* **2009**, *44*, 479–488.

(22) Gomez-Hernandez, A.; Sanchez-Galan, E.; Ortego, M.; Martin-Ventura, J. L.; Blanco-Colio, L. M.; Tarin-Vincente, N.; Jimenez-Nacher, J. J.; Lopez-Bescos, L.; Egido, V.; Tunon, J. Effect of intensive atorvastatin therapy on prostaglandin E2 levels and metalloproteinase-9 activity in the plasma of patients with non-ST-elevation acute coronary syndrome. *Am. J. Cardiol.* **2008**, *102*, 12–18.

(23) Axelrad, T. W.; Kakar, S.; Einhorn, T. A. New technologies for the enhancement of skeletal repair. *Injury* **2007**, *38* (Suppl.), 549–562.

(24) Coutant, K. D.; Wolff-Winiski, B.; Ryder, N. S. Fluvastatin enhances receptor-stimulated intracellular $Ca2^{+}$ release in human keratinocytes. *Biochem. Biophys. Res. Commun.* **1998**, *245*, 307–312.

(25) Miller, S. C.; Marks, S. C., Jr. Local stimulation of new bone formation by prostaglandin E1: quantitative histomorphometry and comparison of delivery by minipumps and controlled-release pellets. *Bone* **1993**, *14*, 143–151.

(26) Blackwell, K. A.; Raisz, L. G.; Pilbeam, C. C. Prostaglandins in bone: bad cop, good cop?. *Trends Endocrinol. Metab.* **2010**, *21*, 294–301.