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Original Article

Hylonome

Regulation of osteoclast differentiation by statins

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Abstract

HMG-CoA reductase inhibitor (statin) treatment is frontline therapy for lowering plasma cholesterol levels in patients with hyperlipidemia. In a few case studies, analysis of clinical data has revealed a decreased risk of fracture in patients on statin therapy. However, this reduction in the incidence of fracture is not always observed nor is it supported by an increase in bone density, which further complicates our understanding of the role of statins in bone metabolism. Thus, the precise role of statins in bone metabolism remains poorly understood. In this study, we examined the effect of statin treatment on osteoclastogenesis. Treatment with lovastatin resulted in a significant, dose-dependent decrease in the numbers of differentiated osteoclasts and decreased cholesterol biosynthesis activity with an EC_{50} similar to that observed in freshly isolated rat or cultured human liver cells. Studies assessing the role of mevalonate metabolites in the development of the osteoclasts demonstrated that geranylgeraniol, but not squalene or farnesol was important for the development and differentiation of osteoclasts, implicating protein geranylgeranylation rather than protein farnesylation as a key factor in the osteoclast differentiation process. In conclusion, our data indicate that lovastatin inhibits osteoclast development through inhibition of geranylgeranylation of key prenylated proteins and that the bone effects of statins are at least partially due to their effects on osteoclast numbers.

Keywords: Osteoclast, Statin, Lovastatin, Geranylgeraniol

Introduction

The enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase mediates cholesterol biosynthesis by converting HMG-CoA to mevalonic acid (Figure 1). Statins have been shown to be reversible, competitive inhibitors of HMG-CoA reductase[1-4] and are widely used in the treatment of hyperlipidemia. Statins, in addition to their beneficial lipid-lowering effects, have been shown to have pleiotropic effects[5-7] in various systems such as the nervous system[8], the immune system[9,10], the cardiovascular system[7,11-13], and most recently the skeletal system[7,14,15]. In addition, statins have been shown to have potential for anti-cancer therapy[16] and as immunosuppressors in organ transplantation[17,18]. The mechanisms by which these effects in diverse tissues occur are still not well understood and have only recently begun to be investigated.

Recently, preclinical animal studies[7,14,15] and case-controlled observational clinical studies[19-23] have suggested that statins may have beneficial effects on the skeleton. Statins block the conversion of HMG-CoA to mevalonic acid (Figure 1), while bisphosphonates, potent bone resorption inhibitors, have been shown to block the formation of geranylgeranyl and farnesyl diphosphates. Inhibition of the prenylation of key GTP-binding proteins prevents the formation of the osteoclast ruffled border, inhibiting resorption and ultimately leading to apoptosis of osteoclasts. Experimental evidence from preclinical animal studies has shown that statins promote bone formation[7,14,15]. In these studies, direct injection of lovastatin or simvastatin over the calvaria of mice induced new bone formation, while rats that have lost bone due to ovariectomy (OVX) showed an increase in their bone formation rates and trabecular bone volume after systemic treatment with simvastatin[7,14] Similarly, evidence from *in vitro* studies showed that simvastatin treatment induced mRNA expression of bone morpho-

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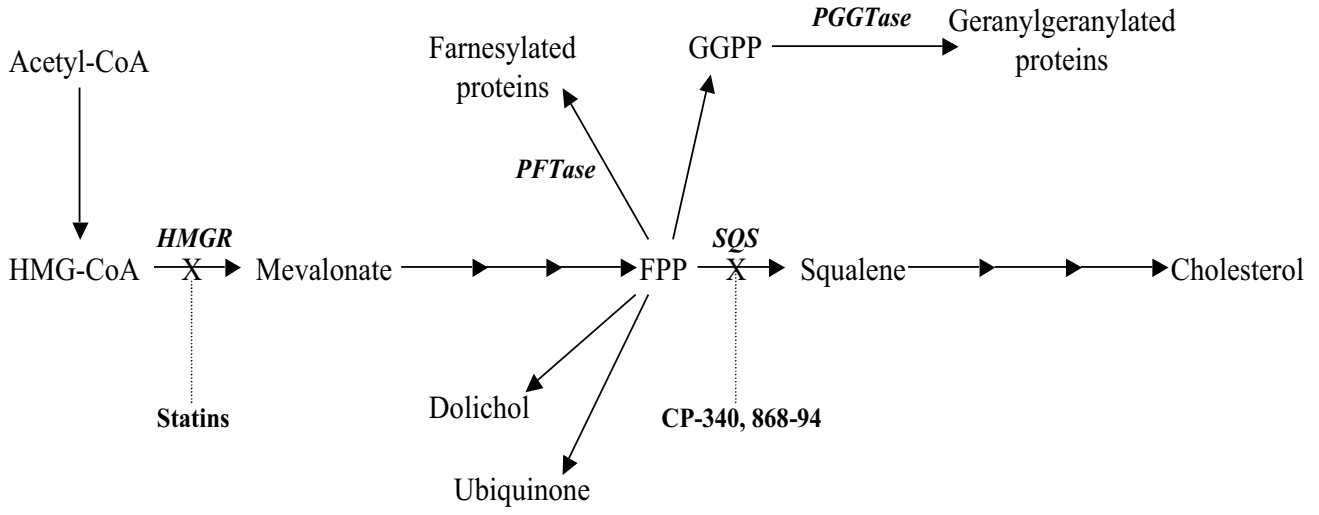

Figure 1. Cholesterol and polyisoprenoid pathway. HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; HMGR, HMG-CoA reductase; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; PFTase, protein farnesyltransferase; PGGTase, protein geranylgeranyltransferase; SQS, squalene synthetase; Statins, lovastatin, simvastatin, pravastatin, atorvastatin, etc.; CP-340,868-94, N- (-)-[(3R, 5S)-7-chloro-5- (1-naphthyl)-1-neopentyl-2-oxo-1, 2,3,5-tetrahydro-4, 1-benzothiazepin-3-acetyl] isonipecotic acid, L-arginine trihydrate salt.

genetic protein-2 (BMP-2) in human MG63 cells and mouse MC3T3-E1 osteoblastic cells. This induction of BMP-2 was suggested to be the mechanism-of-action of statins in bone[7,14,24]. Retrospective analysis of clinical studies with statins has shown that the incidence of fractures was reduced by 50% in patients who were taking statins[21,22] while in other studies bone mineral density (BMD) was significantly increased in patients receiving statin therapy[19,25]. However, data from double-blinded placebo controlled clinical trials in osteoporotic patients are not available nor is this decrease in fracture risk observed in all clinical studies[26,27]. Reanalysis of the LIPID study[28] could find no effect of statins on fracture risk. Several published reports using preclinical animal models also showed no effect on fracture risk or BMD in animals after statin treatment[29,30]. In addition, studies comparing markers of bone formation and bone resorption in control and statin treated patient populations have had varying outcomes[31-35]. Because of these variable outcomes in both preclinical and clinical studies, the role of statins in bone metabolism is poorly understood.

To date, the treatment of osteoporosis, a disease characterized by a reduction in bone mass and strength due to the destruction of bone architecture which ultimately leads to increased risk of fracture[36], remains a major challenge even though a wide variety of therapeutic agents has been developed. These therapies include hormone replacement therapy (HRT), calcitonin, selective estrogen receptor modulators (SERMs) and bisphosphonates. However, few therapeutic agents have been found that can restore the lost bone. The putative bone anabolic activity observed with statins makes them attractive potential therapeutic agents for the treatment of osteoporosis in an aging population if their bone anabolic effects can be confirmed.

Because the precise role of statins in maintaining and or improving skeletal integrity remains poorly understood, we sought to determine if the reduced fracture risk reported in some studies upon statin therapy was due to a reduction in bone resorption. Other antiresorptive agents such as calcitonin, for example, when administered intranasally, showed a 3% increase in lumbar BMD and a 30% drop in vertebral compression fractures[37,38] whereas raloxifene, when administered orally, showed a 3% increase in lumbar BMD and a 30% drop in vertebral fractures[39]. In order to address this question, we refined an *in vitro* osteoclast differentiation system that uses marrow from OVX rats[40]. The OVX rat is a widely accepted preclinical model for estrogen deficiency-induced bone loss. After surgical induction of estrogen deficiency by ovariectomy, rat skeletons exhibit an increase in osteoclast number that eventually leads to the loss of trabecular bone[41,42]. We have previously reported an increase in the number of osteoclasts formed *in vitro* when rat bone marrow was derived from OVX rats[40]. Presumably, this increase in osteoclast number is preceded by an increase in the number of pre-osteoclastic cells in the bone marrow. The recent discovery of osteoprotegerin (OPG) and its ligand, receptor activator of NF-κB ligand (RANKL), has aided our understanding of osteoclast biology through the ability of these proteins to induce differentiation of bone marrow cells to osteoclasts *in vitro*. OPG is a naturally occurring secreted protein with homology to members of the TNF receptor

family that inhibits *in vivo* osteoclastogenesis and blocks the increase in osteoclast numbers seen in osteopenic disorders[43]. Its ligand, RANKL, activates mature osteoclasts and modulates osteoclast formation from bone marrow precursors[44]. By the addition of macrophage-colony stimulating factor (M-CSF) and RANKL, also known as osteoprotegerin ligand (OPGL), TNF-related activation-induced cytokine (TRANCE) or osteoclast differentiation factor (ODF), osteoclast production can be enhanced *in vitro*[43-46]. We have utilized this cell culture system to characterize the role of lovastatin on osteoclast differentiation. Here we report that lovastatin treatment of *in vitro* rat bone marrow cell cultures decreases the numbers of osteoclasts produced after six days of culture in a dose-dependent manner that parallels its reduction in cholesterol biosynthesis, and present evidence that this reduction in osteoclast formation is likely due to a reduction in key protein geranylgeranylation.

Materials and methods

In vitro rat bone marrow culture

The *in vitro* rat bone marrow culture system was adapted from previously published methods[40,47-50]. Briefly, female Sprague Dawley (SD) rats (2.5 to 3 months of age, 240-280 grams; Taconic, Germantown, NY) are bilaterally ovariectomized. Grasser et al.[40] have previously shown an increase in osteoclast number after OVX; however, similar results were observed using bone marrow from normal wild type rats (data not shown). Two weeks after surgery, tibiae were removed from euthanized animals and cut on a diamond wafer blade (#11-4244 Buehler, Lake Bluff, IL) at points just below the epiphyseal growth plate of the proximal tibia and above the tibio-fibular junction. The marrow from the tibiae of 10 rats was flushed out of the bone into complete tissue culture media phenol red-free α-Minimum Essential Media (α-MEM) with 15% fetal bovine serum (FBS) and antibiotics (Invitrogen, Carlsbad, CA). Exuded marrow pieces were triturated approximately 20 times using a wide bore transfer pipette and the cell suspension was filtered through a 100-μm-mesh cell strainer (Falcon #2360, Becton Dickinson Co., Franklin Lakes, NJ) to remove bone fragments and vascular remnants. The cell suspension was brought up to 50 mls with complete media, counted on a hemacytometer and plated at 1 x 10^5 cells/cm^2 in 24-well plates in complete media containing 25 ng/ml each of RANKL and M-CSF (R & D Systems, Minneapolis, MN) and 10^{-8} M 1, 25-dihydroxyvitamin D_3 (Biomol, Plymouth Meeting, PA) and cultured in 5% CO2 at 37˚C. A 50 mM aqueous stock of lovastatin [Mevinolin, (Sigma/Aldrich, St Louis, MO)] was prepared by dissolving 23 mg of lovastatin in 434 μl of ethanol and then adding 62 μl of 1.0 N sodium hydroxide. After a two-hour incubation of the suspension at 37˚C (which reduces lactone to free acid), 742 ul of deionized distilled water was added. Further dilutions were made from this stock. Marrow cultures were fed by replacing one-half of the media with fresh complete media on day three and the cultures (n=8 wells) were dosed with either vehicle (0.1% ethanol final concentration) or lovastatin at 10^{-8} M, 10^{-10} M, 10^{-12} M final concentrations. The marrow cultures were fed and dosed again on day 5, and on day 6 were stained for TRAP (tartrate-resistant acid phosphatase) using the leukocyte acid phosphatase kit (Sigma/Aldrich, St. Louis, MO). Following TRAP staining, the number of TRAP (+) cells having >3 nuclei were counted as osteoclasts.

For experiments using mevalonate pathway intermediates, a squalene synthetase inhibitor, CP-340,868-94 (N- (-)-[(3R, 5S)-7-chloro-5- (1-naphthyl)-1-neopentyl-2-oxo-1, 2,3,5-tetrahydro-4, 1-benzothiazepin-3-acetyl] isonipecotic acid, L-arginine trihydrate salt; rat liver enzyme inhibition IC_{50} = 28 nM; rat primary hepatocyte cholesterol synthesis inhibition EC_{50} = 100 nM; Hep-G2 (human hepatoma) cell cholesterol synthesis inhibition EC_{50} = 600 nM; IM-9 (human lymphoblast) cell cholesterol synthesis inhibition EC_{50} = 70 nM [51] was prepared at a stock concentration of 10 mM in dimethylsulfoxide (DMSO) and diluted to the desired final compound concentrations of 0.3 μM and 3 μM in culture media. Geranylgeraniol (Sigma/Aldrich, St Louis, MO) was solubilized in methanol containing 10mM ammonium hydroxide, and stock solutions of farnesol (Sigma/Aldrich, St Louis, MO) and geranylgeraniol were made at 10 mM in ethanol before addition to the cultures at a final concentration of 2.5 μM (final concentration of DMSO in all cultures was 0.1%).

To confirm that osteoclasts produced in our *in vitro* culturing system had the requisite cell phenotype, we performed a series of experiments.

Calcitonin Labeling

First, to confirm the presence of calcitonin receptors, we performed calcitonin labeling and autoradiography. Rat bone marrow cells were cultured as described above in 4-well chamber slides (Nunc, Inc., Naperville, IL) for six days. Before incubation with [^{125}I] - Calcitonin (2000 Ci/mmol, Amersham Pharmacia Biotech, Piscataway, NJ), cells were rinsed with α-MEM containing 0.1% BSA for 10 minutes at room temperature (RT). Cultures were incubated with 0.2 nM [^{125}I] - Calcitonin in α-MEM containing 0.1% BSA plus or minus 200 nM salmon calcitonin (BACHEM California, Torrance, CA) for 1 hour at RT. After labeling, the cells were rinsed with cold α-MEM, fixed with 2% paraformaldehyde in 0.1 M sodium cacodylate (Electron Microscopy Sciences, Fort Washington, PA) for 10 minutes, washed with α-MEM (2 x 5 min) and TRAP stained. Slides were then dipped into Kodak NTB-2 autoradiography emulsion, exposed in the dark for three weeks at 4˚C and developed in D-19 developer diluted 1:2 with water for 4 minutes at 16ºC. After fixation the slides were rinsed and mounted in glycerol/PBS (90:10 v/v) for viewing under an Olympus BH-2 microscope (Olympus America, Inc., Melville, NY).

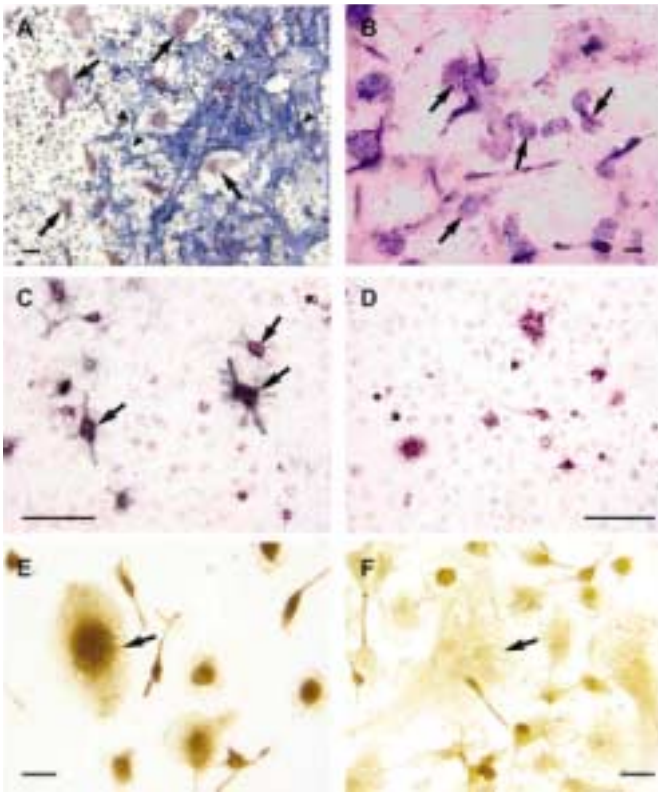

Figure 2. Osteoclast differentiation in cultures of rat bone marrow cells. TRAP histochemical staining, resorption pit formation, calcitonin receptor binding and cathepsin K immunolocalization confirmed the differentiation of rat marrow cultured in the presence of RANKL, M-CSF and Vitamin D_3 into functional osteoclasts. (A) TRAP staining of six-day cultures. TRAP (+) cells with three or more nuclei (arrows) are shown. Alkaline phosphatase staining was also performed to indicate osteoblastic cell types (arrowheads). (B) Resorption lacunae on bovine bone slices. The presence of resorption lacunae indicates functional osteoclasts (several are indicated by arrows) are produced after 8 days of culture. (C) Calcitonin receptor localization with TRAP staining. Silver grains can be seen over TRAP (+) multinucleated cells of six day cultures (arrow). (D) No silver grains can be seen over TRAP (+) cells after incubation with excess of unlabeled calcitonin. (E) Cytoplasmic immunolocalization of cathepsin K. Cathepsin K was observed in osteoclasts (arrow) and some mononuclear cells of 6-day rat marrow cultures using an anti-peptide (carboxy terminus) affinity-purified goat polyclonal antibody. (F) The same cultures incubated with pre-immune serum demonstrate a lack of specific staining (arrow). Magnification bars are 25 μm (A, B), 100 μm (C, D), 50 μm (E, F).

Cathepsin K immunolocalization

Because cathepsin K has been shown to be highly expressed in human osteoclasts, we wanted to know if cathepsin K was expressed in our cells. Therefore, six-day-old marrow cultures were prepared in chamber slides (Becton Dickinson Co., Franklin Lakes, NJ) and cultured as described above and fixed with 4% paraformaldehyde in calcium-magnesium free phosphate-buffered saline (CMF-PBS), pH 7.2 for 20 minutes at RT and rinsed in CMF-PBS (3 x 5 minutes). Endogenous biotin activity was blocked using 3% hydrogen peroxide in methanol (30 minutes). After rinsing in CMF-PBS, 0.1% BSA (10 minutes at RT) and CMF-PBS, 0.1% BSA, 0.1% Triton X-100 (10 minutes at RT), slides were blocked with 1.5% horse serum in CMF-PBS (30 minutes). Cells were incubated in an affinity-purified goat polyclonal cathepsin K antibody (10 μg/ml, Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C in a humid chamber. Specific antibody labeling was visualized using a diaminobenzidine (DAB)-based immunoenzymatic detection method according to manufacturer's protocols (Vector Laboratories, Burlingame, CA).

Resorption pit formation on bone slices

To confirm that the multinucleated cells formed in culture had the ability to resorb bone, we used the resorption pit assay as previously described by Sato and Grasser[52]. Briefly, marrow cells (5 x 10^5 cells/cm^2) were plated onto bovine bone slices (4.5 mm^2, 100 μm thick) in 96-well plates and cultured as described above with one-half of the medium being replaced every three days. Bone slices were collected at 8, 10, 12 and 14 days of culture, placed in distilled water and sonicated (3 x 30 sec) at RT. Bone slices were rinsed in ethanol, air dried, stained with toluidine blue and examined for the presence of resorption pits under reflected light.

Incorporation of [^{14}C] acetate into [^{14}C] cholesterol in marrow cultures

Subconfluent rat bone marrow cell cultures were prepared as described above and plated in T-75 flasks. On day 3, cultures were treated with either vehicle (0.1% ethanol) or vehicle containing lovastatin at 1 nM, 3 nM, 30 nM, 100 nM and 300 nM final concentrations and incubated at 37°C for 1 hour followed by the addition of [2^{-14} C] acetate (86 mM, 129 dpm/pmol; NEN, Boston, MA). After 6 hours incubation in the presence of radiolabeled acetate, the medium from the flasks was transferred to 50-ml tubes. Two ml of 0.1 N sodium hydroxide was added to each flask to dissolve the cell monolayers, and the dissolved cell suspension was combined with the medium. To each 12 ml sample was added 12 ml of ethanol, 2 ml 50% potassium hydroxide, and 50,000 cpm of [1,2^{-3}H] cholesterol as an internal recovery standard. The samples were saponified at 90°C for 2 hours, cooled to RT and extracted 3 times with 20 ml hexane. The pooled hexane extracts were evaporated to dryness under nitrogen, redissolved in 50 ml chloroform-hexane (1:1 v/v), spotted on plastic-backed silica gel 60 TLC plates (Eastman Kodak, Rochester, NY), and developed in hexane-diethyl ether-acetic acid (70:30:2 v/v/v). The region of the chromatogram

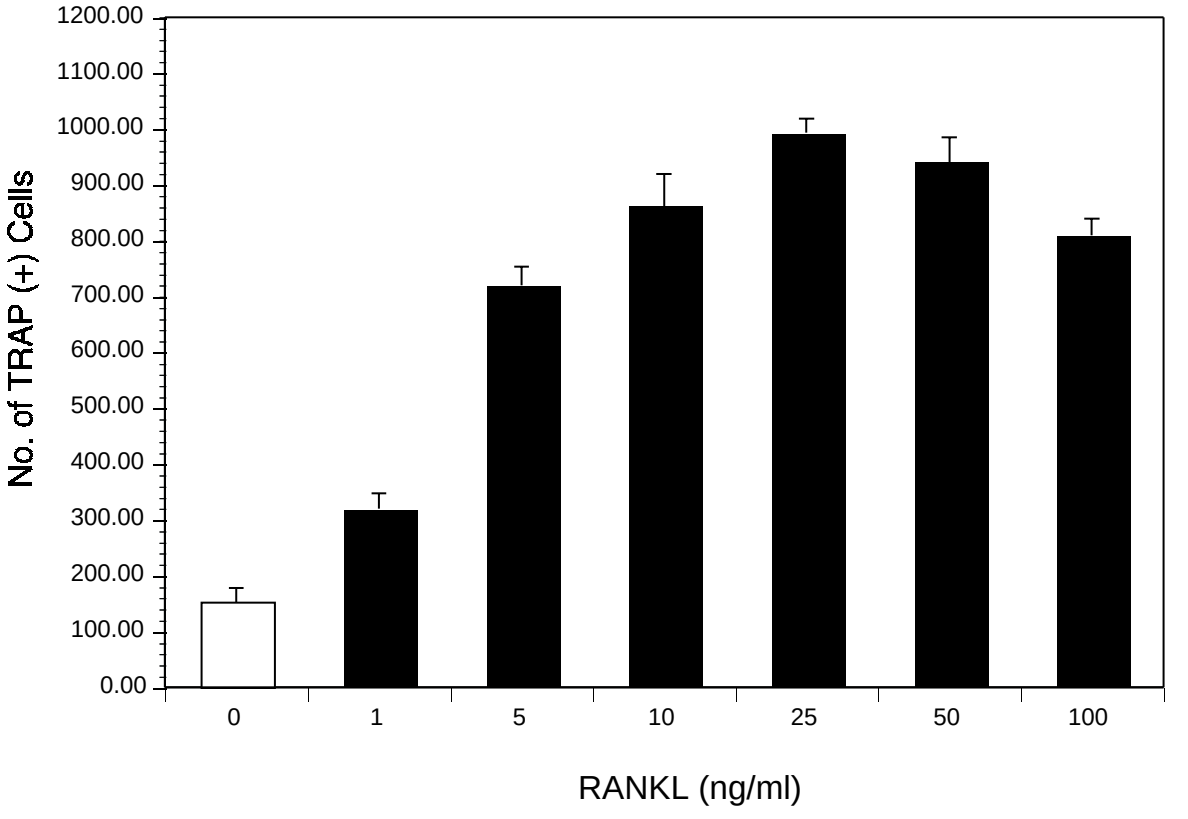

Figure 3. Dose response with RANKL. Rat bone marrow cells were plated out as described in Materials and methods and cultured for six days with 1 to 100 ng/ml of RANKL, 25 ng/ml M-CSF and 10^{-8} M Vitamin D_3. The cultures were fixed and stained for the presence of TRAP. The numbers of TRAP (+) cells with three or more nuclei were counted. Our results indicate that 25 ng/ml RANKL was the optimum concentration for obtaining an average of ~1000 cells per well. Similar observations were made in two independent experiments with each bar representing the mean of 4 individual wells. The data is represented as the mean + SEM.

corresponding to cholesterol mobility was cut out, placed in 6 ml of Aquasol-2 (NEN, Boston MA), and counted in a Beckman LS 6500 liquid scintillation counter. Results are expressed in cpm, corrected for recovery[53].

Statistical analysis

Statistics were calculated using StatView 4.0 software (Abacus Concepts, Inc., Berkeley, CA) and are expressed as mean ± SEM for each group. The analysis of variance (ANOVA) test followed by Fisher's protected least significant difference was used to compare the differences between the groups.

Results

In order to study the effect of statins in bone metabolism, we used rat bone marrow cells obtained from OVX rats to generate osteoclasts. Rat bone marrow cells were cultured in the presence of 25 ng/ml each of RANKL and M-CSF and 10^{-8} M 1, 25-dihydroxyvitamin D_3 to produce multinucleated TRAP (+) cells (Figure 2A). These cells were able to excavate resorption lacunae on bovine bone slices (Figure 2B), were calcitonin receptor expression positive (Figures 2C, D) and were cathepsin K immunoreactive (Figures 2E, F). These properties taken together confirm that we are able to differentiate rat bone marrow cells into osteoclasts.

The concentration of RANKL used in our rat marrow cell cultures was optimized by culturing the marrow cells in the presence of various concentrations of RANKL ranging from 1 ng/ml to 100 ng/ml in the presence of 25 ng/ml M-CSF and 10^{-8} M 1, 25-dihydroxyvitamin D_3. Treatment of rat marrow cell cultures with RANKL dose-dependently increased the numbers of osteoclasts at concentrations up to 25 ng/ml when compared to control cultures lacking RANKL (Figure 3). The number of osteoclasts produced in these cultures increased between 2 and 6-fold at concentrations between 1 ng/ml and 25 ng/ml RANKL when compared to control cultures. At concentrations above 25 ng/ml RANKL, no further increase in osteoclast number was observed (Figure 3).

Next, we used these cultures to characterize the role of statins on osteoclastogenesis. Three-day marrow cell cultures were treated with lovastatin at concentrations ranging

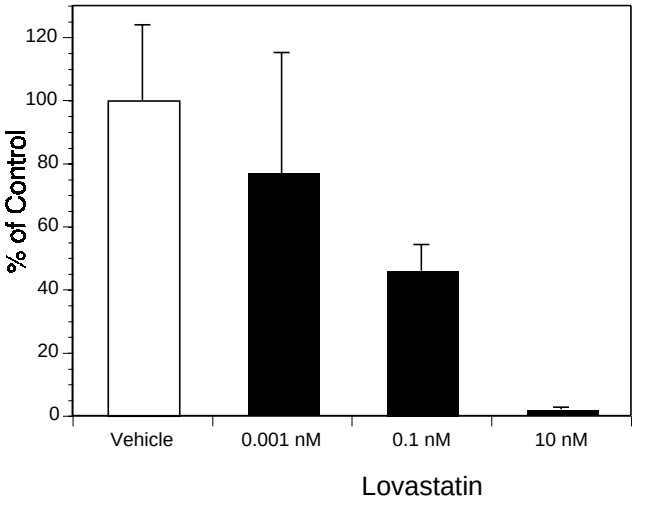

Figure 4. Dose-dependent inhibition of osteoclastogenesis by lovastatin. Multinucleated TRAP (+) cells from rat bone marrow cultures were quantitated after 4 days of treatment with different doses of lovastatin. Similar observations were made in two independent experiments and each bar is the mean of 8 individual wells. The data is represented as % of control + SEM.

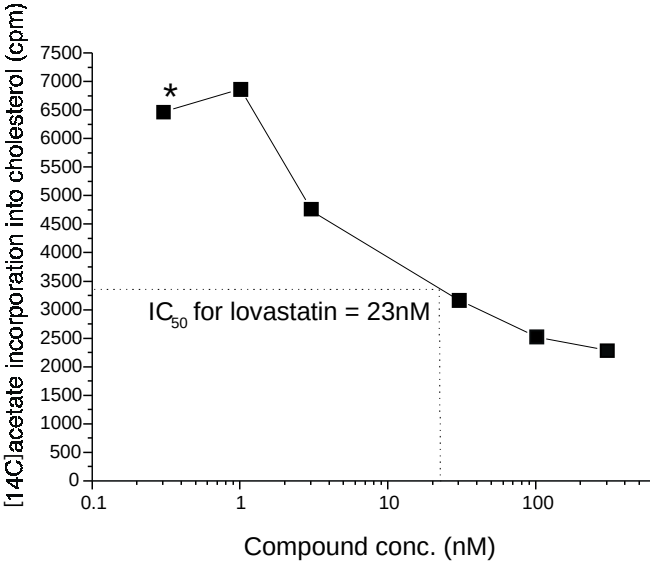

Figure 5. Lovastatin decreases sterol synthesis in cultured rat bone marrow cells. Cholesterol synthesis was measured in rat bone marrow cultures by [^{14}C]-Acetate incorporation into total sterols following a 6-hour pulse. The IC50 of lovastatin is approximately 20 nM. (*; Vehicle)

from 10^{-8} M to 10^{-12} M and the numbers of osteoclasts were determined on day six. Similar experiments were done where marrow cells were treated on day 1 and day 3 (data not shown). A number of experiments were carried out to ensure that the effect of statins on osteoclasts was not due to cell toxicity. At the concentrations of lovastatin used in our experiments (10^{-8} M to 10^{-12} M) no evidence of the loss cell viability was seen using trypan blue staining, whereas, 10^{-6} M lovastatin resulted in a loss of cell viability. The quantitation of TRAP (+) cells in these cultures revealed a dose-dependent decrease in the number of osteoclasts after treatment with lovastatin (Figure 4) when compared to vehicle treated cultures with an EC_{50} for the effect of approximately 10^{-10} M (Figure 4). Similarly, other statins such as mevastatin and lovastatin have been shown to reduce osteoclast number in murine bone marrow cultures[56,61].

Statins are known to inhibit HMG-CoA reductase activity, which prevents the conversion of HMG-CoA to mevalonic acid and ultimately leads to a reduction in the amount of cholesterol synthesized (Figure 1)[53]. At higher doses, statins also reduce nonsterol polyisoprenoid production and protein prenylation (Figure 1)[54]. Because a decrease in the number of osteoclasts *in vitro* after lovastatin treatment was observed, we wanted to establish that there was a corresponding decrease in cholesterol production. Therefore, rat bone marrow cells were again cultured for three days and then treated with lovastatin at 1 nM, 3 nM, 30 nM, 100 nM and 300 nM for one hour. Cholesterol synthesis was measured in rat bone marrow cell cultures by incorporation of [^{14}C] acetate into [^{14}C] cholesterol (see Materials and methods). Quantitation of the [^{14}C] cholesterol revealed a dose dependent decrease in the amount of cholesterol synthesized in our marrow cell cultures when compared to vehicle treated cultures with an EC_{50} of 23 nM (Figure 5). These data indicate that both the cholesterol biosynthetic pathway and osteoclast number are affected upon treatment of rat bone marrow cells with lovastatin. However, the precise mevalonate metabolite that is required for osteoclastogenesis still remained to be elucidated. It has been previously reported that the bisphosphonate, alendronate, inhibits the prenylation of proteins by the mevalonate metabolite geranylgeranyl diphosphate (GGPP), and this reduction in protein geranylgeranylation is responsible for the decrease in osteoclast number observed upon alendronate treatment[55-57].

The loss of prenylation of key proteins after statin treatment could account for the decreased numbers of osteoclasts observed in our experiments. To identify the mevalonate metabolite(s) that are responsible for the inhibition of osteoclastogenesis upon lovastatin treatment, we next tested the ability of CP-340,868-94 (an inhibitor of squalene synthetase; rat liver enzyme inhibition IC_{50} = 28 nM [51]) to affect osteoclast number. A reduction in osteoclast number after CP-340,868-94 treatment would indicate requirement of a post-squalene sterol in the process of osteoclastogenesis (see Figure 1), whereas no effect of CP-340,868-94 on osteoclastogenesis would suggest an effect of lovastatin on osteoclast production via its effects on either nonsterol polyisoprenoid production or on protein isoprenylation (see Figure 1). Rat bone marrow cell cultures were treated on day three of culture with either vehicle (0.1% DMSO) or CP-340,868-94 at 0.3 μM or 3 μM in place of lovastatin, and cultured for another three days.

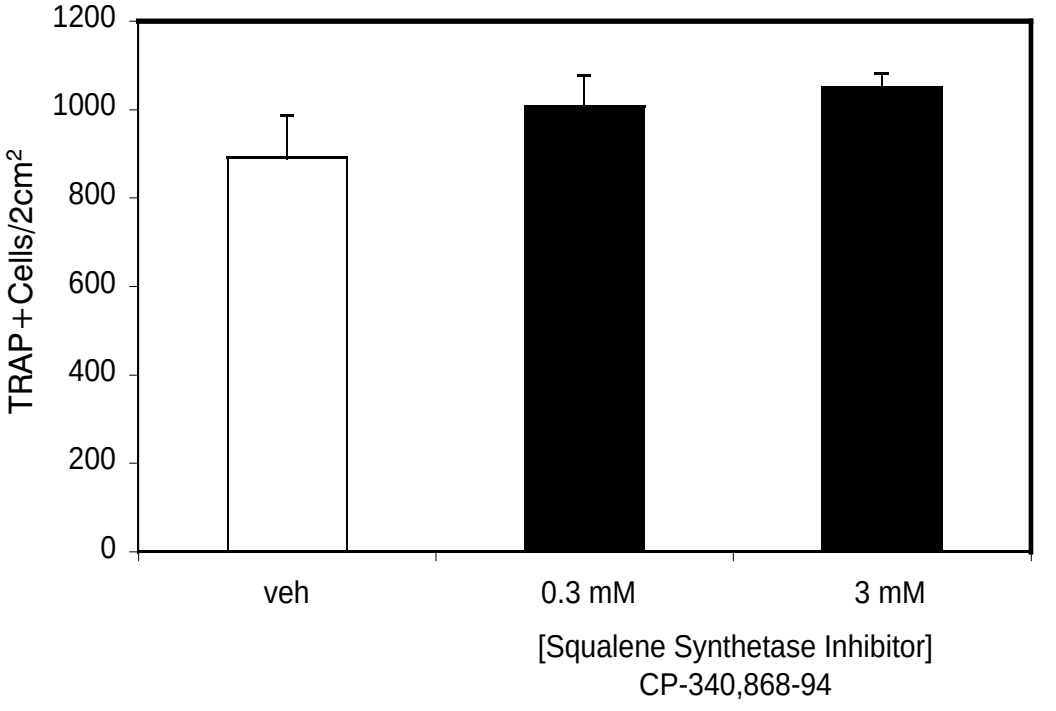

Figure 6. Inhibitory effects of lovastatin are upstream of the pathway intermediate squalene. To determine if metabolites downstream of squalene are necessary for osteoclast formation, an inhibitor of squalene synthetase (CP-340,868-94; IC_{50} = 28 nM) was added to bone marrow cultures in lieu of lovastatin to see if the number of multinucleated TRAP (+) cells would be affected. Each bar represents the mean of eight individual data points and is represented as the mean + SEM.

Quantitation of the number of TRAP (+) cells showed no significant difference in the numbers of osteoclasts upon treatment with either concentration of CP-340,868-94 (Figure 6). Since CP-340,868-94 inhibits cholesterol production in rat primary hepatocytes, Hep-G2 cells, and IM-9 cells with respective EC_{50} values of 100 nM, 600 nM, and 70 nM[51], the lack of effect of CP-340, 868-94 on osteoclast formation at concentrations of 0.3 μM and 3 μM strongly suggests that the decrease in osteoclast number upon lovastatin treatment is not due to a decrease in either post-squalene sterol production or cholesterol concentration, but rather is due to the reduction of a presqualene pathway intermediate.

To further identify the mevalonate metabolite involved in the reduction in osteoclast number and to determine the role of protein prenylation in osteoclastogenesis, lovastatin treated marrow cell cultures were also treated with the key mevalonate metabolite precursors, farnesol and geranylgeraniol. Both isoprenoid alcohols are converted intracellularly to their respective pathway intermediate diphosphates, farnesyl diphosphate (FPP) and geranylgeranyl diphosphate (GGPP)[58,59]. In these experiments, three-day rat marrow cell cultures were treated with vehicle, lovastatin (25 nM) or lovastatin (25 nM) plus either farnesol (2.5 μM) or geranylgeraniol (2.5 μM); cultured for three additional days and the numbers of TRAP (+) cells were quantitated. As can be seen in Figure 7, farnesol had no effect in restoring the lovastatin-induced reduction in osteoclast number while the addition of geranylgeraniol significantly restored ($p < 0.001$) the osteoclast number when compared to lovastatin. These results preclude roles either for protein farnesylation or for key FPP-derived nonsterol polyisoprenoids, such as dolichol and ubiquinone, in the mechanism of osteoclastogenesis (see Figure 1), but rather, demonstrate that the inability to geranylgeranylate key proteins can account for the reduced osteoclast numbers, confirming that geranylgeranylation is required for osteoclast development. It should be noted however, that although FPP can be converted to GGPP (Figure 1) at significantly elevated levels, the observation that farnesol treatment did not also reverse the lovastatin inhibition indicates that the concentration of farnesol used in these experiments was not sufficient to allow for conversion of FPP to GGPP.

Discussion

We report here that rat bone marrow cells cultured in the presence of RANKL, M-CSF and 1, 25-dihydroxyvitamin D_3 will differentiate into cells that are phenotypically osteoclasts. Treatment of these marrow cell cultures with lovastatin dose-dependently decreased the numbers of osteoclasts (IC_{50} ~0.01 nM; Figure 4) and the amount of cholesterol synthesized (IC_{50} of 23 nM; Figure 5). The IC50 for cholesterol synthesis inhibition by lovastatin is similar to that measured in

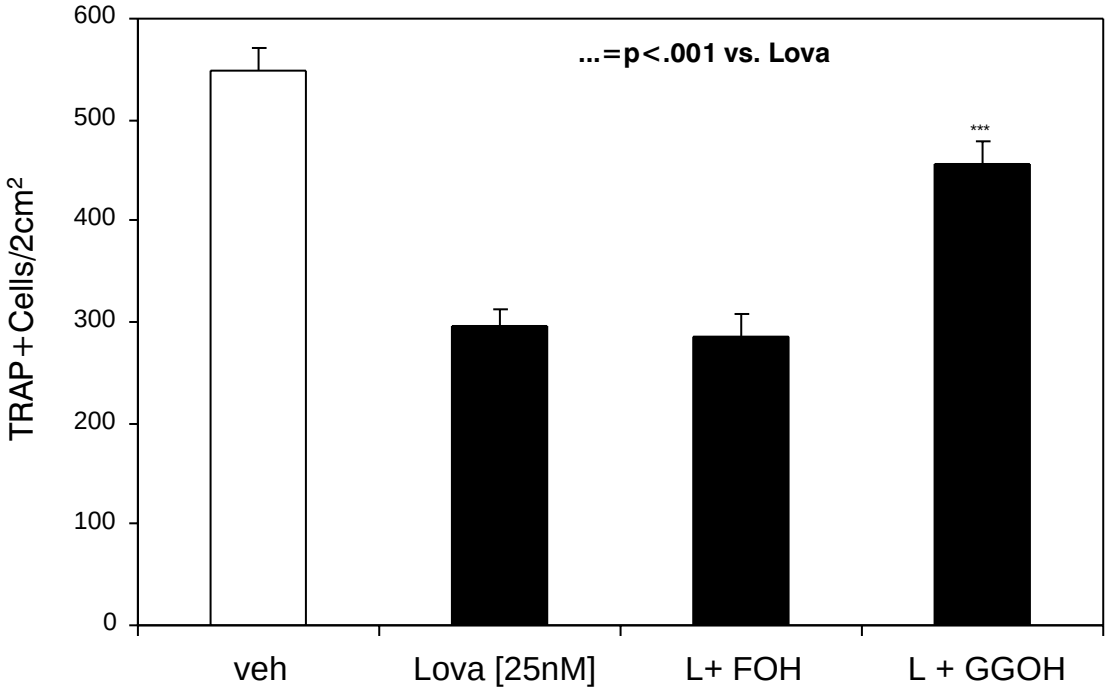

Figure 7. Lovastatin-induced inhibition of osteoclastogenesis is restored by geranylgeraniol but not farnesol. To assess the role of protein prenylation in osteoclastogenesis, key mevalonate metabolite precursors (farnesol and geranylgeraniol) were added to lovastatin ("Lova" or "L")-treated rat bone marrow cultures at 2.5 μM. Both isoprenoid alcohols are converted intracellularly to their respective pyrophosphate pathway intermediates. Farnesol had no effect, but geranylgeraniol significantly ($p<.001$ compared to lovastatin) restored the number of multinucleated TRAP (+) cells as compared with lovastatin-treated cultures. Each bar represents the mean of eight individual data points and is represented as the mean + SEM.

HepG2 cells, which has been reported to be ~20 nM[53] and is consistent with the values of direct enzyme assays of 11 nM [60]. The differences between IC50 values for cholesterol synthesis and the decrease in osteoclast number are likely due to differences between acute (1 hour; cholesterol synthesis) and chronic (6 day; osteoclastogenesis) treatment with lovastatin; use of a homogeneous cell line (HepG2) as compared to the mixed cell background of the bone marrow cultures; and the possibility that less than 50% inhibition of cholesterol synthesis is sufficient to induce osteoclastogenesis inhibition. We have further shown that treatment of these rat marrow cultures with a squalene synthetase inhibitor did not affect osteoclast numbers. This indicates that the decrease in osteoclast number upon lovastatin treatment is not due to a decrease in post-squalene sterol production or cholesterol concentration, but rather is due to the reduction of a presqualene pathway intermediate. This was confirmed when geranylgeraniol but not farnesol was able to significantly restore osteoclast numbers in cultures that had been treated with lovastatin, suggesting the importance of protein geranylgeranylation in the osteoclastogenic pathway. Geranylgeranylation of specific proteins, potentially key GTP-binding proteins (e.g., rho, rab, rac proteins) involved in actin cytoskeleton regulation, apoptosis, membrane ruffling and vesicular trafficking, play a role in osteoclast development and function[55,61,62]. Because osteoclasts are very dynamic cells, the disruption of any of these processes has been shown to result in decreased numbers of osteoclasts or impairment of osteoclast function[56]. Further study of the mechanism by which geranylgeranylated proteins regulate osteoclast function will be the subject of future experimentation.

In conclusion, the data presented here shows that treatment with lovastatin results in a dose-dependent decrease in osteoclast numbers. Our results agree with the decreases seen in dynamic parameters (osteoclasts/mm^2 of bone surface) by Mundy et al.[14] after oral administration of statins to intact or ovariectomized rats. The evidence for the bone effects of statins in humans has come from observational studies and not randomized trials. These studies have shown increases in BMD and reductions in the incidence of fracture risk. Studies using animal models or human clinical samples where parameters such as bone turnover rates are measured have not been done to date. It has been reported that inhibitors of bone resorption such as calcitonin[37,38], raloxifene[39] and alendronate[63] all have slight effects on BMD while showing substantial effects on fracture reduction. Our data suggest that statins may be reducing fracture risk as a result of inhibition of resorption rather than a stimulation of bone anabolism as previously suggested.

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