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Dose-Specific Biphasic Effects of Simvastatin on the Expression of CXCL10 and CX3CL1 by Human Osteoblasts

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Abstract

Objective: To better understand the effects of simvastatin (SS) on the expression and secretion of two chemokines, CXCL10 and CX3CL1, by osteoblasts, and to test whether inhibition of isoprenoid intermediates of cholesterol biosynthesis were involved in the effects of SS.

Methods: Human osteoblasts were incubated in the presence or absence of the inflammatory cytokines tumor necrosis factor alpha (TNF- α) and interferon gamma (IFN- γ), with and without SS (0.1–100 μ M). Culture supernatants were then collected, and expression of CXCL10 and CX3CL1 mRNA in osteoblasts was examined using quantitative TaqMan real-time polymerase chain reaction. The levels of CXCL10 and CX3CL1 were measured using enzyme-linked immunosorbent assays.

Results: At a high concentration (100 μ M), SS inhibited expression and secretion of the chemokines and showed cytotoxicity, whereas at lower concentrations (0.1–1 μ M) SS stimulated the expression and secretion of the chemokines. Expression and secretion of CXCL10 or CX3CL1 from osteoblasts were induced by stimulation with TNF- α and IFN- γ . In addition, SS exerted a biphasic effect on the evoked induction of CXCL10 and CX3CL1. Chemokine expression and secretion was also assayed in the presence of mevalonate (MEV), geranylgeranyl pyrophosphate (GGPP) or farnesyl pyrophosphate (FPP). MEV abolished both the inhibitory effect of high-dose SS and the stimulatory effect of low-dose SS. On the other hand, GGPP abolished only the inhibitory effects of high-dose SS, and FPP had no effect at all.

Conclusions: These findings suggest that osteoblasts are an important cellular source of CXCL10 and CX3CL1, and that statins such as SS may modulate the inflammatory process in bone tissues to inhibit bone resorption and stimulate bone formation through biphasic modulation chemokine synthesis.

Keywords: chemokine, CXCL10, CX3CL1, osteoblasts, statin

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Introduction

Chemokines and proinflammatory cytokines appear to play key roles in a variety of immune/inflammatory responses.^{1,2} The maintenance of skeletal homeostasis is a dynamic process driven by the coordinated cellular activities of osteoblasts (OBs), osteocytes and osteoclasts.³ Moreover, cells involved in bone formation (e.g. OBs) express a variety of chemokines that likely play key roles during bone remodeling under both normal and pathological conditions.⁴ For example, we and others have shown that CX3CL1 (fractalkine) and CXCL10 (interferon-inducible protein 10) are expressed and secreted by OBs.^{5–7}

Inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase inhibitors (statins) are widely used for lowering serum cholesterol concentrations, and provide an important and effective approach to the treatment of hyperlipidemia and arteriosclerosis.⁸ In addition, several recent studies have shown that statins such as simvastatin (SS) also affect inflammation and immune function by modulating the expression and function of inflammatory molecules, including cytokines and metalloproteinase expressed by endothelial cells and macrophages.^{9–11} Furthermore, it was recently reported that statins modulate the inflammatory process in bone tissues and osteogenic cells, thereby inhibiting bone resorption, stimulating bone formation, and modulating angiogenic factor synthesis.^{12–15} Since osteoblast-derived chemokines have important roles in during bone remodeling under both normal and pathological conditions, in the present study we examined the expression and secretion of CXCL10 and CX3CL1 by OBs and their modulation by SS.

Materials and Methods

Reagents

Complete medium consisted of Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% heat-inactivated fetal bovine serum (FBS; Gibco Laboratories, Grand Island, NY). Tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) were purchased from Genzyme/Techne (Cambridge, MA). SS was kindly provided by Merck & Co., Inc, Whitehouse Station, NJ. For experimentation, SS was dissolved in ethanol and converted to the open

acid form. To test whether inhibition of isoprenoid intermediates of cholesterol biosynthesis is involved in the effect of SS, chemokine secretion was also examined in the presence of mevalonate (MEV; 100 µM, Sigma Aldrich, St. Louis, MO), geranylgeranyl pyrophosphate (GGPP; 10 µM; Sigma-Aldrich) and farnesyl pyrophosphate (FPP; 10 µM; Sigma-Aldrich).

Preparation of human osteoblasts

Human OBs were purified from metaphyseal trabecular bones collected from proximal femora of post-traumatic patients ($n = 3$) during total hip arthroplasty, as described previously.¹⁶ Briefly, after removing pieces of cortical bone, articular cartilage and soft connective tissue, the fragments were cut into small pieces and incubated with DMEM containing 1% collagenase for 30 min at 37 °C, after which they were extensively washed. The resultant bone explants were plated in tissue culture plates and allowed to grow in DMEM containing 10% FBS. Once cell outgrowths from bone explants were confluent (after 3–5 weeks of culture), the explants were removed, and the cells were trypsinized, and replated into new 48-well (experiments for protein secretion) or 6-well (for mRNA expression) culture plates at a density of 1×10^5 /ml. The obtained cells exhibited a flattened polygonal shape with multiple spindlelegs as well as features of the OB-like phenotype, including osteocalcin expression, bone alkaline phosphatase expression, and mineralization as determined by von Kossa staining (data not shown). The cells were incubated for 3 days with medium alone, thereafter the indicated cytokines were added, with or without SS, and the conditioned culture medium was collected after 24 hours incubation for protein assays and after 4 hours incubation for mRNA experiments. All human experiments were performed in accordance with protocols approved by the Human Subjects Research Committee at our institution, and informed consent was obtained from all patients.

Evaluation of cytotoxicity

The effect of SS on cell viability was evaluated based on trypan blue exclusion and release of lactate dehydrogenase (LDH). For trypan blue assays, the indicated concentrations of SS were added to OBs, after which the cells were then incubated for up to 24 h, detached using trypsin, and resuspended in 1 ml

of DMEM. Trypan blue solution (Gibco, Grand Island, NY, USA) was then added to a final concentration of 0.2% and incubated for 1 min. Cells positive for trypan blue were counted using a hemocytometer under a light microscope (Nikon) and were considered dead. In addition, the amount of LDH released into the culture medium was determined using an automatic autoanalyzer system (Hitachi, Tokyo, Japan).

Enzyme-linked immunosorbent assays

CXCL10 and CX3CL1 were quantified using double ligand enzyme-linked immunosorbent assays (ELISAs) that were modifications of an assay described previously.^{17,18} Monoclonal murine anti-human CXCL10 (1 µg/ml; Genzyme/Techne, Cambridge, MA) or CX3CL1 (4 µg/ml; Genzyme/Techne) served as the primary Ab, and biotinylated polyclonal goat anti-CXCL10 (0.1 µg/ml; Genzyme/Techne) and anti-CX3CL1 (0.25 µg/ml; Genzyme/Techne) served as the respective secondary Abs. The sensitivity limit for the CXCL10 and CX3CL1 ELISAs were ~50 pg/ml and ~150 pg/ml, respectively.

Isolation of total RNA and real-time polymerase chain reaction (PCR)

Total RNA was extracted from human OBs using TRIzol reagent (Invitrogen, Carlsbad, CA), after which 1-µg samples were reverse transcribed into cDNA by incubation with TaqMan RT reagents (Applied Biosystems, Foster City, CA), first for 120 min at 37 °C and then for 10 min at 25 °C. Real-time PCR was carried out using an ABI Prism® 7900 sequence detection system (Applied Biosystems). The PCR mixture included 40 ng of cDNA, which was amplified using AmpliTaq Gold DNA polymerase (Applied Biosystems) as described previously.¹⁶ Appropriate Assays-on-Demand™ primers and probes (Applied Biosystems) were used for detection of CXCL10, CX3CL1 and ribosomal RNA (rRNA) expression. For quantification, the number of target mRNA copies per rRNA copy was calculated, and values were expressed as fold increases over control (culture medium alone).

Statistical analysis

Data are expressed as means ± SEM. Groups were compared using analysis of variance, after which the means of groups whose variances were determined

to significantly differ were compared using Student's *t*-test for comparison of means of multiple groups. Values of *p* < 0.05 were considered significant.

Results

Effects of SS on cell viability

We, first, carried out trypan blue dye exclusion assays and measured LDH activity in culture supernatants to determine the degree to which cytotoxicity contributed to the inhibitory effects of higher concentrations of SS. After stimulating OBs with IFN-γ (1000 IU/ml) + TNF-α (20 ng/ml), treatment with SS at concentrations ranging from 0.1–10 µM elicited no significant changes in the numbers of viable (trypan blue negative) cells or LDH activity, though small increases and decreases were noted (Table 1). At a concentration of 100 µM, however, SS significantly reduced the numbers of viable cells and increased release of LDH.

Effects of simvastatin on expression and secretion of OB-derived CXCL10 and CX3CL1

We next determined whether secretion of CXCL10 and CX3CL1 from OBs could be induced by the inflammatory cytokines TNF-α and IFN-γ. We found that high-dose (1000 IU/ml) of IFN-γ induced secretion of small amounts of CXCL10 or CX3CL1 from OBs, as compared with medium alone, but TNF-α had no effect. On the other hand, when applied together, the two cytokines appeared to act synergistically to induce significant secretion of both CXCL10 and CX3CL1 from OBs (Figs. 1 and 2), which is consistent with earlier findings by us and others.^{7,19}

Next, OBs stimulated with IFN-γ+TNF-α were incubated with various concentrations of SS, and CXCL10 and CX3CL1 in the culture supernatants were assayed. As shown in Figure 1A, a low concentration of SS (0.1 µM) significantly enhanced secretion of CXCL10 from OBs stimulated with IFN-γ + TNF-α. At a higher SS concentration (100 µM), however, the cytokine-induced secretion of CXCL10 was significantly inhibited. In addition, similar induction (0.1µM SS) and inhibition (10 and 100 µM SS) of CXCL10 were seen in IFN-γ-stimulated OBs (Fig. 1B). SS also exerted this biphasic effect on CX3CL1 secretion from OBs stimulated by IFN-γ + TNF-α (Fig. 2A) or IFN-γ alone (Fig. 2B), though



Table 1. Effect of SS on cell viability of OBs.

Conditions	TNF- α + IFN- γ						
	SS (μ M)						
	-	0.01	0.1	1	10	100	100
Cell numbers ($\times 10^5$)	3.5 \pm 1.0	3.2 \pm 0.7	3.9 \pm 0.8	2.9 \pm 0.9	3.0 \pm 1.4	1.7 \pm 0.6*	3.1 \pm 0.5**
LDH (IU/l)	45.9 \pm 10.3	51.0 \pm 5.1	48.0 \pm 11.9	45.4 \pm 2.8	69.6 \pm 15.6	152.9 \pm 39.6*	63.1 \pm 9.1**

OBs (1×10^5 cells/ml) were stimulated with TNF- α (20 ng/ml) plus IFN- γ (1000 u/ml) for 24 h, with and without the indicated doses of SS, after which trypan blue exclusion and LDH release were assayed. Also examined were the effects of MEV, GGPP and FPP. To determine OB-derived total LDH, the cells were lysed by addition of the same volume of complete medium, followed by three freeze/thaw cycles. LDH concentrations were then determined to be 286.0 ± 24.5 IU/l. Three separate experiments were performed using three different OB samples. Data are expressed as the mean \pm SEM. * $p < 0.05$ vs. medium alone. ** $p < 0.05$ vs. 100 μ M SS.

only the inhibitory effect of 100 μ M IFN- γ was statistically significant.

To test whether the inhibitory effects of SS on secreted CXCL10 and CX3CL1 antigen levels were accompanied by corresponding changes in the levels of CXCL10 and CX3CL1 mRNA in OBs, we used real-time PCR to assess levels of chemokine mRNA. As shown in Figure 3, the expression of both CXCL10 and CX3CL1 mRNA was enhanced in OBs by lower concentrations of SS (0.1–1 μ M), though only the effect on CXCL10 was significant. Higher concentrations of SS (10–100 μ M) significantly inhibited expression of CXCL10 and CX3CL1 mRNA.

Effects of MEV, GGPP and FPP on the regulation of chemokine expression

To assess the extent to which intermediates created during cholesterol biosynthesis were involved in the observed effects of SS, chemokine expression and secretion were also examined in the presence of MEV (100 μ M), GGPP (10 μ M) or FPP (10 μ M), three inhibitors of different steps in the cholesterol biosynthetic pathway. MEV abolished the inhibitory effect of high-dose (100 μ M) SS and the stimulatory effect of low-dose (0.1 μ M) SS on cytokine (TNF- α + IFN- γ)-evoked expression and secretion of CXCL10 (Figs. 4A and B). MEV had similar effects on the expression and secretion of CX3CL1, though some of the changes were not statistically significant (Figs. 5A and B). Notably, GGPP had no significant effect on the stimulation of CXCL10 expression secretion by low-dose SS; however, it abolished the inhibitory effect of high-dose SS (Figs. 4A and B). In contrast to MEV or GGPP, FPP had no effects on chemokine expression or secretion at any dose. Finally, the reduction in cell viability induced by high-dose SS was abolished by MEV and GGPP, but not by FPP (Table 1).

Discussion

The results of the present study suggest that OBs are prominent cellular sources of both CXCL10 and CX3CL1, which are secreted in response to stimulation by TNF- α and/or IFN- γ . Furthermore, SS exerted a biphasic effect on OB-derived CXCL10 and CX3CL1 synthesis: at higher concentrations, SS inhibited the expression and secretion of the chemokines and exerting a cytotoxic effect.

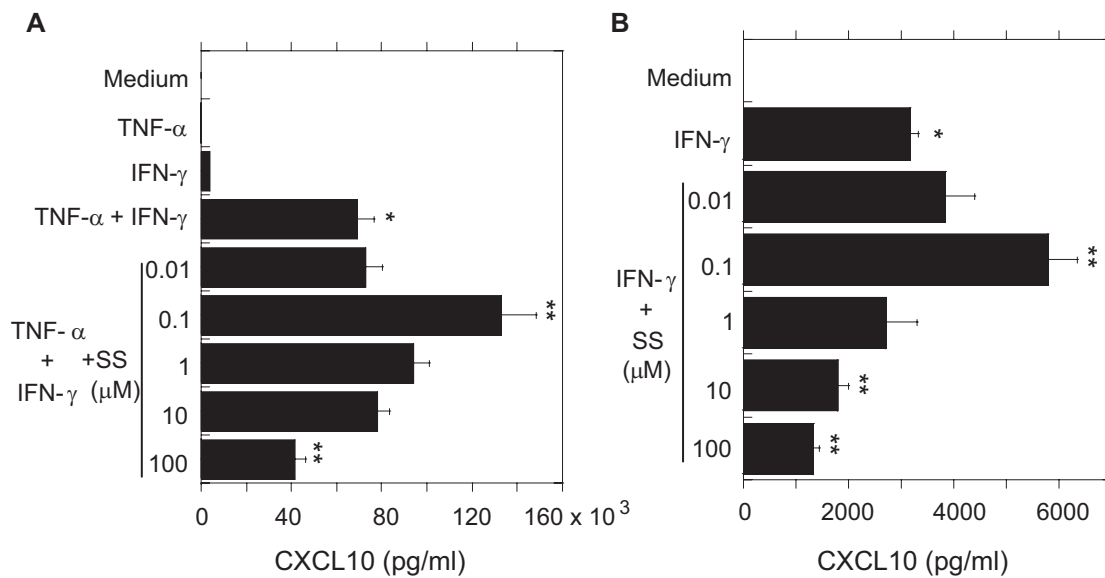


Figure 1. Effects of SS on secretion of CXCL10 from OBs stimulated with TNF- α and/or IFN- γ . OBs were incubated for 24 h with TNF- α (20 ng/ml) + IFN- γ (1000 IU/ml) (A) or IFN- γ alone (B), with or without the indicated concentrations (0.01–100 μ M) of SS. CXCL10 levels in the culture supernatants were assayed using a specific ELISA. Data are expressed as the means \pm SEM of three independent experiments. * p < 0.05 vs. control medium. ** p < 0.05 vs. cytokine-stimulated OBs.

In particular, the negative effect of 100 μ M SS on the mRNA expression of both chemokines seemed to be related to the inhibition of chemokine gene specific regulation, although the decreased secretion of both chemokines was, in part, dependent upon the drug's cytotoxicity at this high-dose of SS (100 μ M).

While at lower concentrations it stimulated both the expression and secretion of the chemokines.

Consistent with our present results, it was recently reported that statins exert a dose-dependent, biphasic effect on cultured ECs and angiogenesis.^{20,21} In addition, 10 μ M SS significantly increased the secretion of IL-1 β

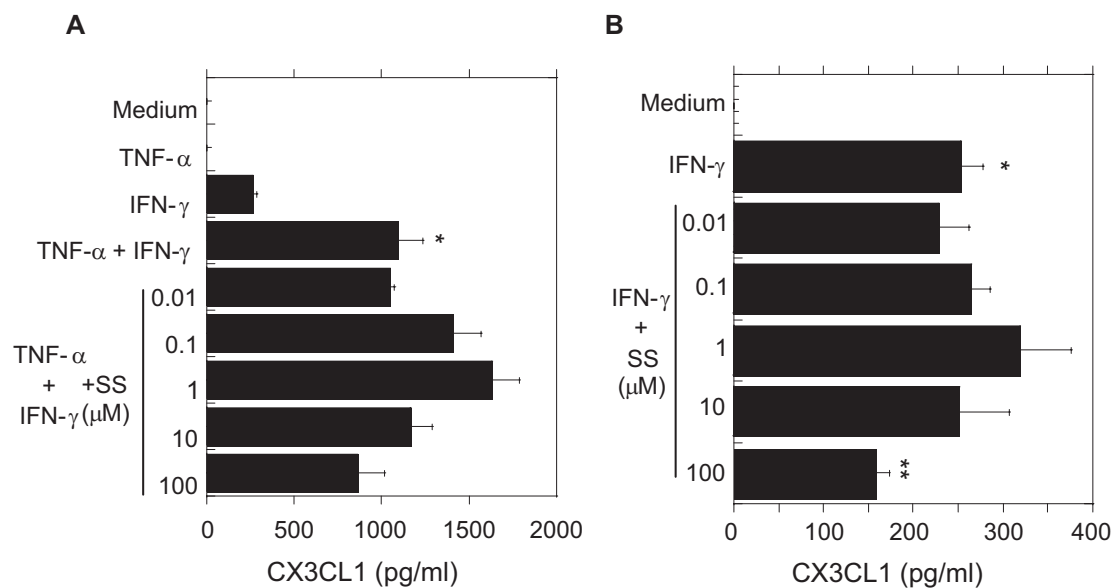


Figure 2. Effects of SS on secretion of CX3CL1 from OBs stimulated with TNF- α and/or IFN- γ . OBs were incubated for 24 h with TNF- α (20 ng/ml) + IFN- γ (1000 IU/ml) (A) or IFN- γ alone (B), with or without the indicated concentrations (0.01–100 μ M) of SS. CX3CL1 levels in culture supernatants were assayed using a specific ELISA. Data are expressed as the means \pm SEM of three independent experiments. * p < 0.05 vs. control medium. ** p < 0.05 vs. cytokine-stimulated OBs.

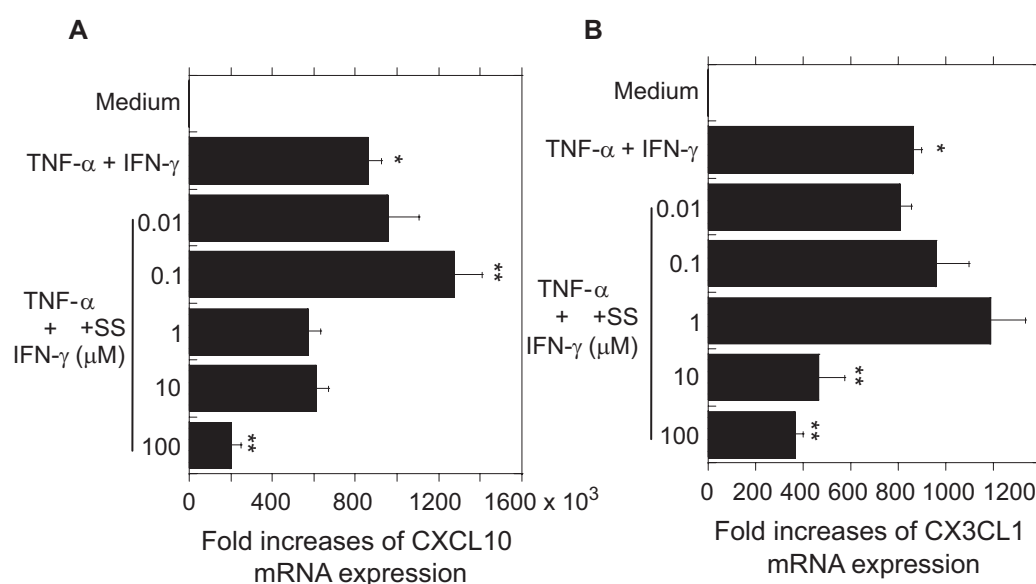


Figure 3. Effects of SS on cytokine-induced expression of CXCL10 and CX3CL1 mRNA in OBs. OBs were incubated for 4 h with TNF- α (20 ng/ml) + IFN- γ (1000 IU/ml) with or without the indicated concentrations of SS, after which expression of CXCL10 (A) and CX3CL1 (B) mRNA was assayed using real-time PCR. For quantification, the number of target mRNA copies per rRNA copy was calculated, and values were expressed as fold increases over control (culture medium alone). Data are expressed as the means \pm SEM of three independent experiments. * p < 0.05 vs. control medium. ** p < 0.05 vs. cytokine-stimulated OBs.

from macrophages,²² while 0.1–10 μ M SS increased expression of vascular endothelial growth factor (VEGF) by osteoblastic cells (MC3T3-E1 cells) and induced osteoblastic differentiation of untransformed MC3T3-E1 osteoblasts.^{14,23} On the other hand, 1 μ M SS inhibited secretion of IL-6 and IL-8 from

IL-1 α -stimulated epithelial cells,²⁴ 5–50 μ M SS inhibited secretion of matrix metalloproteinases from IL-1 β -stimulated human chondrocytes,¹¹ and 0.1–10 μ M SS inhibited secretion of IL-6 or IL-8 from TNF- α -stimulated fibroblast-like synoviocytes.²⁵ These inhibitory effects of statins appear to be dependent on

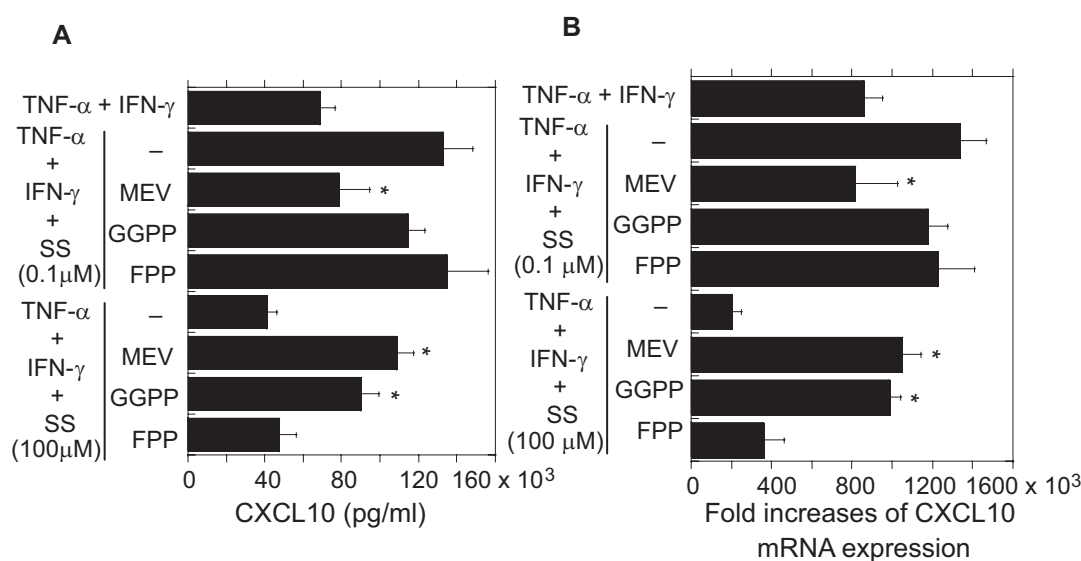


Figure 4. Effects of MEV, GGPP and FPP on CXCL10 secretion (A) and mRNA expression (B) in cytokine-stimulated OBs treated with SS. OBs were incubated for 24 h (for cytokine secretion) or 4 h (for cytokine mRNA expression) with TNF- α (20 ng/ml) + IFN- γ (1000 IU/ml) and the indicated concentrations of SS. Effects of MEV (100 μ M), GGPP (10 μ M) or FPP (10 μ M) on CXCL10 secretion (A) and mRNA expression (B) were assessed. Data are means \pm SEM from three independent experiments. * p < 0.05 vs. TNF- α /IFN- γ -stimulated OBs treated with SS.

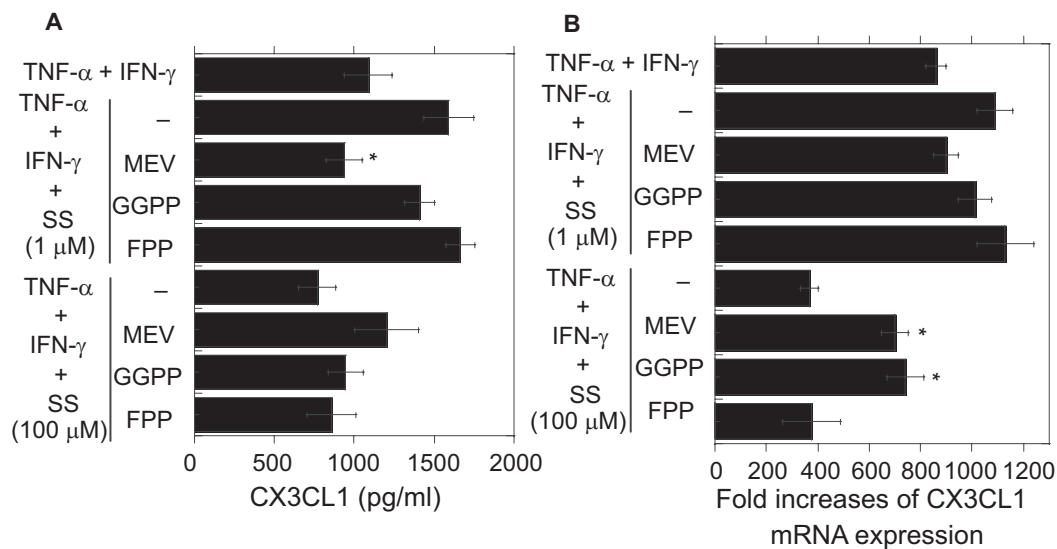


Figure 5. Effects of MEV, GGPP and FPP on CX3CL1 secretion (A) and mRNA expression (B) from cytokine-stimulated OBs treated with SS. OBs were incubated for 24 h or 4 h with TNF- α (20 ng/ml) + IFN- γ (1000 IU/ml) and the indicated concentrations of SS. The effects of MEV (100 μ M), GGPP (10 μ M) or FPP (10 μ M) on CX3CL1 secretion (A) and mRNA expression (B) were assessed. Data are means \pm SEM from three independent experiments. * p < 0.05 vs. TNF- α /IFN- γ -stimulated OBs treated with SS.

cytotoxic effects of high-dose SS,²⁶ which is consistent with our finding that high-dose SS reduces synthesis of OB-derived chemokines by exerting a cytotoxic effect.

The OB cytotoxicity, and thus the inhibitory effects, of high-dose SS was reversed by MEV or GGPP, but not FPP, while the stimulatory effects of low-dose SS were reversed by MEV, but not by GGPP or FPP. That MEV reversed the biphasic effect of SS on chemokine expression in OBs confirms the involvement of the cholesterol synthesis pathway in the modulation of chemokine expression. Moreover, our finding that GGPP reversed the inhibitory effect of high-dose SS on chemokine synthesis, but not the stimulatory effect of low-dose SS, suggests the latter is not dependent on the membrane anchorage of small G proteins (e.g. Rho) or the G γ subunit of heterotrimeric G proteins, as both are regulated by GGPP prenylation.²⁷ In addition, our finding that FPP had no effects indicates the biphasic response to SS is not mediated via farnesylation. It has been shown elsewhere that geranylgeranylated and farnesylated Rho family GTPases are involved in regulating cellular functions, including the synthesis of some cytokines and chemokines.^{28,29} It would be interesting to explore the signal transduction pathway via which SS modulates the induction of inflammatory proteins in OBs.

There is also a discrepancy between our findings and those of Wu et al, who reported that alkaline phosphatase activity and calcific nodule formation were stimulated by 0.1–0.4 μ M SS in an osteoblast cell line (M2–10B4 cells), although these concentrations of SS inhibited both calcific nodule formation and alkaline phosphatase activity in aortic valve myofibroblasts.³⁰ The lowest concentration of SS (0.1 μ M) used in the present study is similar to the serum levels measured in patients' receiving regular doses of SS for therapy (usually less than 90 nM).^{31,32} Given our present results and the earlier findings of others, one might expect a clinical dose of SS to increase chemokine secretion from OBs at sites of bone inflammation, though it remains unclear whether higher doses of SS are cytotoxic to bone tissues *in vivo*.

In conclusion, several recent investigations, as well as our present study, have noted that statins such as SS modulate the inflammatory process in bone tissues, thereby inhibiting bone resorption, stimulating bone formation, and biphasically modulating chemokine synthesis. A more complete understanding of the mechanisms by which statins affect OB-derived chemokine (e.g. CXCL10 and CX3CL1) synthesis under pathological conditions such as rheumatoid arthritis, as well as under normal conditions, should provide new strategies for clinical intervention.



Conflict of Interest Statement

T. Kasama received research grants from Banyu Pharmaceuticals Inc.

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