Simvastatin antagonizes tumor necrosis factor- α inhibition of bone morphogenetic proteins-2-induced osteoblast differentiation by regulating Smad signaling and Ras/Rho-mitogen-activated protein kinase pathway

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Abstract

Recent studies have shown that the mevalonate pathway plays an important role in skeletal metabolism. Statins stimulate bone morphogenetic proteins-2 (BMP-2) production in osteoblasts, implicating a possible beneficial role for statins in promoting anabolic effects on bone. Here, we investigated the effects of a lipophilic simvastatin on osteoblast differentiation using mouse myoblast C2C12 cells, in the presence of tumor necrosis factor- α (TNF- α), an inflammatory cytokine that inhibits osteogenesis. The addition of TNF- α to C2C12 cells suppressed the BMP-2-induced expression of key osteoblastic markers including Runx2 and alkaline phosphatase (ALP) activity. Simvastatin had no independent effects on Runx2 and alkaline phosphatase activity; however, it reversed the suppressive effects of TNF- α . The ability of simvastatin to reverse TNF- α inhibition of BMP-induced Smad1,5,8 phosphorylation and Id-1 promoter activity suggests the involvement of Smad signaling pathway in simvastatin action. In addition, cDNA array analysis revealed that simvastatin increased expression levels of Smads in

C2C12 cells exposed to TNF- α that also activated mitogen-activated protein kinase (MAPK) signaling pathways, including extracellular signal-regulated kinase 1/2 (ERK1/2), P38, and stress-activated protein kinase/c-Jun NH2-terminal kinase (SAPK/JNK). Simvastatin potently suppressed TNF- α -induced phosphorylation of ERK1/2 and SAPK/JNK by inhibiting TNF- α -induced membrane localization of Ras and RhoA. Farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) reversed the simvastatin effects on TNF- α -induced activation of Ras/Rho/MAPK pathways. FPP and GGPP also restored the simvastatin effects on TNF- α -induced suppression of Runx2 and ALP activity. In addition, simvastatin decreased the expression levels of TNF type-1 and -2 receptor mRNAs. Collectively, simvastatin supports BMP-induced osteoblast differentiation through antagonizing TNF- α -to-Ras/Rho/MAPK pathway and augmenting BMP-Smad signaling, suggesting a potential usage of statins to ameliorate inflammatory bone damage.

Journal of Endocrinology (2008) **196**, 601–613

Introduction

Bone morphogenetic proteins (BMPs), members of the transforming growth factor (TGF)- β superfamily, have established critical roles in governing various aspects of embryological development, including brain, heart, kidney, and eyes (Reddi 1997). BMPs also play a pivotal regulatory role in mesoderm induction and dorsoventral patterning of developing limb buds and are known to promote differentiation of mesenchymal stem cells into chondrocytes and osteoblasts as well as the differentiation of osteoprogenitor cells into osteoblasts (Lieberman *et al.* 2002). The biological functions of BMPs are mediated through the Smad signal transduction pathway via BMP receptors. In addition to the

established developmental actions of BMPs, a variety of physiological BMP actions in many endocrine and vascular tissues including the ovary (Otsuka *et al.* 2000, Shimasaki *et al.* 2004), pituitary (Otsuka & Shimasaki 2002), thyroid (Suzuki *et al.* 2005), adrenal (Suzuki *et al.* 2004, Kano *et al.* 2005, Inagaki *et al.* 2006), kidney (Otani *et al.* 2007), and vascular smooth muscle cells (Takeda *et al.* 2004) have been elucidated.

Osteoblasts, which arise from mesenchymal stem cell precursors, undergo differentiation in response to a number of factors including BMPs, TGFs, insulin-like growth factor-I (IGF-I), vascular endothelial growth factor (VEGF), and steroids (McCarthy *et al.* 1989, Noda & Camilliere 1989, Celeste *et al.* 1990, Midy & Plouet 1994, Hughes *et al.* 1995, Goad *et al.* 1996, Gerber *et al.* 1999, Spelsberg *et al.* 1999).

Once matrix synthesis begins in cultured osteoblast cells, they differentiate and osteoblastic markers including alkaline phosphatase (ALP), type-I collagen and osteocalcin are activated. Osteoblasts then embed in the extracellular matrix consisting of collagen fibrils, and the matrix is mineralized and extended in the collagen fibrils. Deposition and maintenance of mineralized skeletal elements are further regulated by various growth factors and cytokines.

Mundy *et al.* (1999) first reported that statins stimulate *in vivo* bone formation in rodents and increase new bone volume in mouse calvaria cell cultures. Statins also stimulate expression of bone anabolic factors, such as VEGF and BMP-2 (Maeda *et al.* 2003), and promote osteoblast differentiation and mineralization in MC3T3-E1 cells derived from new bone mouse calvaria (Maeda *et al.* 2001). Statins are potent inhibitors of cholesterol biosynthesis widely used to reduce serum cholesterol levels in hyperlipidemic patients (Hamelin & Turgeon 1998, Maron *et al.* 2000). Statins inhibit 3-hydroxy-3-methylglutarylcoenzyme A (HMG-CoA) reductase to block the conversion of HMG-CoA to mevalonate, a rate-limiting step in cholesterol synthesis (Goldstein & Brown 1990). By inhibiting the initial part of the cholesterol synthesis pathway, statins decrease the availability of several important lipid intermediate compounds including isoprenoids such as farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP). These compounds are associated with post-translational modification of various small G-proteins including Ras and Ras-like proteins such as Rho, Rac, and Rab (Casey & Seabra 1996), which interact with the downstream mitogen-activated protein kinases (MAPKs) and play a central role in cellular responses such as cell proliferation, apoptosis, migration, and gene expression.

Little is known, however, about cellular mechanisms of statin actions in regulating osteoblast function. The present study was undertaken to investigate changes in marker expression corresponding to stages of osteoblast differentiation in statin-treated C2C12 cells focusing on the effects of a critical inflammatory cytokine, tumor necrosis factor (TNF)- α . The pluripotent mesenchymal precursor cell line C2C12, a subclone of a mouse myoblastic cell line, has been widely used as a model to examine the early stages of osteoblast differentiation during bone formation in muscular tissues (Katagiri *et al.* 1994, Ebisawa *et al.* 1999). Macrophages play a key role in chronic inflammation and joint destruction of rheumatoid arthritis by secreting pro-inflammatory cytokines including TNF- α (Feldmann *et al.* 1996). The clinical effectiveness of blocking TNF- α in treating active rheumatoid arthritis established the pathogenic significance of TNF- α in this disease (Feldmann & Maini 2001, Scott & Kingsley 2006). TNF- α produced by macrophages and inflammatory cells induces apoptosis or necrosis in various other cell types. TNF- α induces osteoclast differentiation leading to excess of bone resorption (Kudo *et al.* 2002). Since bone loss in arthritis is related to the activation of TNF- α system, it can be hypothesized that TNF- α also directly controls osteoblast survival and/or osteoblast function related to bone formation. However, roles of the TNF- α signaling

system in determining osteoblast function and its differentiation remain unsolved.

In the present study, we investigated the effects of a lipophilic simvastatin and TNF- α on osteoblast differentiation using mouse myoblast C2C12 cells. This study shows that simvastatin supports BMP-induced osteoblast differentiation by antagonizing TNF- α -to-MAPK pathway and augmenting BMP-Smad signaling, suggesting a potential usage of simvastatin to ameliorate inflammatory bone damages shown in rheumatoid arthritis.

Materials and Methods

Reagents and supplies

Dulbecco's modified Eagle's medium (DMEM), penicillin-streptomycin solution, dimethylsulfoxide, FPP and GGPP were purchased from Sigma-Aldrich Co. Ltd. Recombinant human TNF- α was obtained from PeproTech EC Ltd (London, UK), and recombinant human BMP-2 was purchased from R&D Systems Inc. (Minneapolis, MN, USA). Simvastatin was provided by Merck & Co. Inc., and converted to the active form by alkaline hydrolysis. Id-1-Luc plasmid was kindly provided from Drs Tetsuro Watabe and Kohei Miyazono, Tokyo University (Japan).

Cell culture

The mouse myoblast cell line C2C12 was obtained from American Type Culture Collection (Manassas, VA, USA). C2C12 cells were cultured in DMEM supplemented with 10% fetal calf serum (FCS) and penicillin-streptomycin solution at 37 °C under a humid atmosphere of 95% air/5% CO₂. Changes in cell morphology were monitored using an inverted microscope.

RNA extraction and quantitative real-time RT-PCR analysis

To prepare total cellular RNA, C2C12 cells were cultured in a 12-well plate (2×10^5 viable cells) and treated with indicated concentrations of BMP-2, TNF- α , simvastatin, and either FPP or GGPP in serum-free DMEM. After 48-h culture, the medium was removed, and total cellular RNA was extracted using TRIzol (Invitrogen Corp.), quantified by measuring absorbance at 260 nm, and stored at -80 °C until assay. The extracted RNA (1.0 μ g) was subjected to an RT reaction using the First-Strand cDNA synthesis system (Invitrogen Corp.) with random hexamer (2 ng/ μ l), reverse transcriptase (200 U), and deoxy-NTP (0.5 mM) at 42 and 70 °C for 50 and 10 min respectively. Oligonucleotides used for RT-PCR were custom ordered from Invitrogen Corp. PCR primer pairs were selected from different exons of the corresponding genes as follows: Runx2, 981-999, and

1291–1310 (from GenBank accession no. NM_009820); TNF type-1 receptor (TNFR1), 931–951 and 1211–1231 (BC052675); TNF type-2 receptor (TNFR2), 142–162 and 1142–1162 (Y14622); and a house-keeping gene, ribosomal protein L19 (RPL19), 373–393 and 547–567 (NM_009078). For the quantification of Runx2, TNFR1, TNFR2, and RPL19 mRNA levels, real-time PCR was performed using LightCycler-FastStart DNA Master SYBR Green I system (Roche Diagnostic Co.) under conditions of annealing at 60–62 °C with 4 mM MgCl₂, following the manufacturer's protocol. Accumulated levels of fluorescence were analyzed by the second derivative method after the melting curve analysis (Roche Diagnostic), and then the expression levels of target genes were standardized by RPL19 level in each sample.

ALP determination

After preculture with serum-free DMEM, cells (1×10^5 viable cells) were treated with indicated concentrations of BMP-2, TNF- α , simvastatin, and either FPP or GGPP in 12-well plates. After 72-h culture, cells were lysed and cellular ALP activity was measured by a fluorometric detection kit using 4-methylumbelliferyl phosphate disodium substrate (Sigma Chemical Co). ALP activity of each sample was normalized by protein concentration.

Western immunoblot analysis

Cells (2×10^5 viable cells) were precultured in 12-well plates in DMEM containing 10% FCS for 48 h. After preculture, the medium was replaced with serum-free fresh medium, and then indicated concentrations of BMP-2, TNF- α , simvastatin, FPP, and GGPP were added to the culture medium. After stimulation with growth factors for indicated periods, the membrane fraction of C2C12 cells was extracted by ProteoExtract Native Membrane Protein Extraction Kit (Calbiochem, San Diego, CA, USA). Cells and membrane fraction were solubilized in 100 μ l RIPA lysis buffer (Upstate Biotechnology Inc., Lake Placid, NY, USA) containing 1 mM Na₃VO₄, 1 mM sodium fluoride, 2% SDS, and 4% β -mercaptoethanol. Total cell lysates and the membrane fraction were then subjected to SDS-PAGE/immunoblotting analysis, as reported previously (Inagaki *et al.* 2006) using anti-Runx2 (S-19) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-actin antibody (Sigma-Aldrich Co. Ltd), anti-phospho-Smad1,5,8 antibody (Cell Signaling Technology Inc., Beverly, MA, USA), anti-phospho- and anti-total-extracellular signal-regulated kinase (ERK)1/2 MAPK antibodies (Cell Signaling Technology Inc.), anti-phospho- and anti-total-P38 MAPK antibodies (Cell Signaling Technology Inc.), anti-phospho- and anti-total-stress-activated protein kinase/c-Jun NH2-terminal kinase (SAPK/JNK) MAPK antibody (Cell Signaling Technology Inc.), and anti-pan-Ras and anti-RhoA antibody (Santa Cruz Biotechnology).

Transient transfection and luciferase assay

C2C12 cells (1×10^5 viable cells) were precultured in 12-well plates in DMEM with 10% FCS for 48 h. The cells were then transiently transfected with 500 ng Id-1-Luc reporter plasmid and 50 ng cytomegalovirus- β -galactosidase plasmid (pCMV- β -gal) using FuGENE6 (Roche Molecular Biochemicals) for 24 h. The cells were pretreated with indicated concentrations of BMP-2 and simvastatin for 24 h in serum-free fresh medium and then incubated with TNF- α . After 24-h culture, the cells were washed with PBS and lysed with Cell Culture Lysis Reagent (Toyobo, Osaka, Japan). Luciferase and β -galactosidase (β -gal) activities of the cell lysate were measured by luminescencer-PSN (ATTO, Tokyo, Japan), as reported previously (Miyoshi *et al.* 2006). The data were shown as the ratio of luciferase to β -gal activity.

cDNA array analysis

Oligo GEArray system (SuperArray Bioscience Corp., Frederick, MD, USA) that includes 113 genes of mouse TGF- β and BMP signaling pathway was used for analyzing the expression pattern of BMP signaling system in C2C12 cells. As we reported previously (Miyoshi *et al.* 2006, Otani *et al.* 2007), extracted total RNAs (2.0 μ g) were used as templates to generate biotin-16-dUTP-labeled cDNA probes according to manufacturer's instruction. The cDNA probes were denatured and hybridized at 60 °C with the cDNA array membranes, which were washed and exposed to X-ray films with use of chemiluminescent substrates. To analyze the array results, we scanned the X-ray film and the image was inverted as grayscale TIFF files. The spots were digitized and analyzed using GEArray analyzer software (SuperArray Bioscience Corp.), and the data were normalized by subtraction of the background as the average intensity levels of plasmid DNA of pUC18. The spots of glyceraldehyde-3-phosphate dehydrogenase and cyclophilin A (PPIA) were used as positive controls to compare the membranes. Using these standardized data, we compared the signal intensity of the membranes using the GEArray analyzer program (SuperArray Bioscience Corp.).

Statistical analysis

All results are shown as mean $\pm$ S.E.M. of data from at least three separate experiments, each performed with triplicate samples. Differences between groups were analyzed for statistical significance using ANOVA (StatView 5.0 software, Abacus Concepts Inc., Berkeley, CA, USA). *P* values < 0.05 were accepted as statistically significant.

Results

We first examined the expression profile of Runx2, also called core-binding factor 1 (Cbfa1), induced by BMP-2 treatment

in C2C12 cells. Runx2 is an important bone-specific transcription factor, which is essential for the differentiation of osteoblasts from mesenchymal precursors (Komori *et al.* 1997, Otto *et al.* 1997). Runx2 can directly stimulate transcription of osteoblast-related genes such as those encoding osteocalcin, type I collagen, osteopontin, and collagenase 3 by binding to specific enhancer regions (Ducy *et al.* 1997). Runx2 mRNA levels were significantly increased in the presence of BMP-2 (100 ng/ml) and attained the highest at 48-h culture condition (Fig. 1A). Runx2 protein levels were also enhanced by BMP-2 treatment in accordance with the time-dependent changes of Runx2 mRNA (Fig. 1B). Simvastatin effects on Runx2 mRNA levels were then characterized in the presence or absence of BMP-2. Simvastatin alone (1–100 μ M) had no effects on endogenous Runx2 expression (Fig. 1C). BMP-2 (100 ng/ml) increased levels of Runx2 mRNA expression 2- to 2.5-fold during 48-h culture. Simvastatin (1–30 μ M) had no significant effects on BMP-2-induced Runx2 mRNA expression although a high concentration (100 μ M) of simvastatin had an inhibitory effect on BMP-2-induced Runx2 expression (Fig. 1C). Based on these results, 1–30 μ M simvastatin was used for the following experiments of C2C12 cells.

We next investigated the effects of simvastatin on TNF- α action, which inhibits BMP-2-stimulated osteogenetic properties. C2C12 cells were cultured with TNF- α and BMP-2 in the presence or absence of simvastatin (3–30 μ M). As shown in Fig. 2A, in the absence of simvastatin, BMP-2-induced Runx2 expression was potently suppressed by TNF- α (10–30 ng/ml) in a concentration-responsive manner. Notably, simvastatin impaired the Runx2 mRNA suppression induced by TNF- α in the presence of BMP-2 (Fig. 2A). To confirm the effect of simvastatin at the protein levels, cellular ALP activity was examined in C2C12 cells. Consistent with the simvastatin effects on Runx2 expression, simvastatin also suppressed TNF- α -induced reduction of ALP activity stimulated by BMP-2 (Fig. 2B). Thus, simvastatin concentration-dependently blocked the TNF- α effects that suppress osteoblastic differentiation elicited by BMP-2.

We further investigated the effects of simvastatin pretreatment on Runx2 expression and ALP activity modulated by TNF- α . Following 24-h pretreatment with simvastatin (1 and 10 μ M), C2C12 cells were cultured with BMP-2 (100 ng/ml) and TNF- α (10 ng/ml). When cells were pretreated with simvastatin, the TNF- α effect suppressing BMP-2-induced Runx2 expression was significantly impaired (Fig. 3). In statin-pretreated cells, the peak response of BMP-2-induced ALP activity was potently enhanced (Fig. 4) while the induction of Runx2 mRNA was not affected (Fig. 3). In addition, the TNF- α suppression of ALP activity induced by BMP-2 was also reversed by pretreatment with simvastatin (Fig. 4). Thus, simvastatin might enhance osteoblast differentiation by regulating Runx2 expression and ALP activity through augmenting BMP-2 actions in C2C12 cells.

Figure 1 Characterization of Runx2 expression in C2C12 cells and simvastatin effects on BMP-induced Runx2 expression. (A) Time-course changes of BMP-2-induced Runx2 mRNA expression. C2C12 cells were cultured in the absence or presence of BMP-2 (100 ng/ml) for 72 h. Total cellular RNA was extracted and subjected to RT reaction. For the quantification of Runx2 and RPL19 mRNA levels, quantitative real-time PCR (qPCR) was performed. The expression level of Runx2 was standardized by RPL19 level in each sample. Results are shown as mean $\pm$ S.E.M. of data from at least three separate experiments, each performed with triplicate samples. * P <0.05 and ** P <0.01 versus control levels. (B) Time-course changes of BMP-2-induced Runx2 protein expression. Cells were incubated in the absence or presence of BMP-2 (100 ng/ml) for 72 h. At each time point, cells were lysed and subjected to SDS-PAGE/immunoblot (IB) analysis using antibodies that detect Runx2 protein (55 kDa) and an internal control actin. The results shown are representative of those obtained from three independent experiments. (C) Simvastatin effects on BMP-induced Runx2 mRNA expression. Cells were treated with simvastatin (1–100 μ M) in the absence or presence of BMP-2 (100 ng/ml) for 48 h. Total cellular RNA was extracted and subjected to qPCR analysis as described above. Results are shown as mean $\pm$ S.E.M. of data from at least three separate experiments, each performed with triplicate samples. * P <0.05 versus control levels.

Figure 2 Simvastatin effects on TNF- α suppression of BMP-induced Runx2 expression and alkaline phosphatase (ALP) activity. (A) Simvastatin effects on TNF- α suppression of BMP-induced Runx2 expression. Cells were treated with simvastatin (3–30 μ M) in the presence of BMP-2 (100 ng/ml) and TNF- α (10 and 30 ng/ml) for 48 h. Total cellular RNA was extracted and subjected to RT reaction. For the quantification of Runx2 and RPL19 mRNA levels, qPCR analysis was performed. The expression level of Runx2 was standardized by RPL19 level in each sample. Results are shown as mean $\pm$ S.E.M. of data from at least three separate experiments, each performed with triplicate samples. * P <0.05 and ** P <0.01 versus simvastatin-free control groups or between the indicated groups. (B) Simvastatin effects on TNF- α suppression of BMP-induced ALP activity. Cells were treated with simvastatin (3–30 μ M) in the presence of BMP-2 (100 ng/ml) and TNF- α (10 and 30 ng/ml) for 72 h. Cells were lysed and cellular ALP activity was measured by fluorometric method. Results are shown as mean $\pm$ S.E.M. of data from at least three separate experiments, each performed with triplicate samples. * P <0.05 and ** P <0.01 versus simvastatin-free control groups or between the indicated groups.

In order to investigate the mechanism by which simvastatin cooperates BMP effects, time-course changes of a key BMP signaling, phospho-Smad1,5,8 proteins were detected by western blotting analysis. As shown in Fig. 5A, BMP-2 stimulated Smad1,5,8 phosphorylation. Neither simvastatin nor TNF- α independently had any effect on the Smad1,5,8 activation (Fig. 5A); however, TNF- α inhibited BMP-2-induced Smad1,5,8 phosphorylation (Fig. 5B). Densitometric analysis of band intensities showed that simvastatin partially

Figure 3 Effects of simvastatin pretreatment on TNF- α suppression of BMP-induced Runx2 expression. After preculture, the cells were pretreated with simvastatin (A: 1 μ M and B: 10 μ M) in combination with BMP-2 (100 ng/ml) for 24 h, and then cells were treated with TNF- α (10 ng/ml) for 48 h. Total cellular RNA was extracted and subjected to RT reaction. For the quantification of Runx2 and RPL19 mRNA levels, qPCR analysis was performed. The expression level of Runx2 was standardized by RPL19 level in each sample. Results are shown as mean $\pm$ S.E.M. of data from at least three separate experiments, each performed with triplicate samples. * P <0.05 and ** P <0.01 versus control groups or between the indicated groups.

reversed Smad1,5,8 inactivation caused by TNF- α (Fig. 5B and C). To confirm this finding, we assessed a BMP target gene Id-1 promoter activity using cells transiently transfected with Id-1-Luc (Fig. 5D). In accordance with western blot data of Smad1,5,8 phosphorylation, Id-1-Luc activity elicited by BMP-2 was significantly suppressed by TNF- α . Importantly, 24-h pretreatment with simvastatin restored the suppression of BMP-2-induced Id-1 transcription caused by TNF- α . These results suggest that simvastatin maintains BMP-Smad signaling in C2C12 cells in spite of the TNF- α effects.

BMP type-I receptors, including ALK-2, ALK-3, and ALK-4; type II receptors, including BMPRII, ActRII, and ActRIIB; and Smad signaling molecules, including Smad1, 2, 3, 4, 5, 6, 7, and 8 were clearly expressed in this cell line as reported earlier by RT-PCR (Mukai *et al.* 2007). ALK-6 is not expressed in C2C12 cells. To investigate the mechanism by which simvastatin maintains the BMP signaling in C2C12 cells, cDNA array analysis in BMP/TGF- β signaling

Figure 4 Effects of simvastatin pretreatment on TNF- α suppression of BMP-induced alkaline phosphatase (ALP) activity. After pre-culture, the cells were pretreated with simvastatin (A: 1 μ M and B: 10 μ M) in combination with BMP-2 (100 ng/ml) for 24 h, and then cells were treated with TNF- α (10 ng/ml) for 72 h. Cells were lysed and cellular ALP activity was measured by fluorometric method. Results are shown as mean $\pm$ S.E.M. of data from at least three separate experiments, each performed with triplicate samples. * P <0.05 and ** P <0.01 versus control groups or between the indicated groups.

molecules was performed using total cellular RNAs extracted from C2C12 cells treated with TNF- α (10 ng/ml) alone and TNF- α (10 ng/ml) plus simvastatin (10 μ M) for 24 h (Fig. 6A). Among the key regulatory genes for BMP-Smad activation in C2C12 cells, the enhanced expression of Smad1, 2, and 3 in the cells treated with simvastatin was noted (Fig. 6B), suggesting that increased Smad expression by simvastatin may be involved in facilitating BMP-Smad1,5,8 activation.

There has been increasing evidence suggesting that TNF- α activates MAPK signaling pathway in various cells including C2C12 cells, although the physiological roles of MAPK activation has yet to be elucidated. As demonstrated in Fig. 7A, BMP-2 alone (100 ng/ml) had no effect on MAPK activation, including the ERK1/2, P38, and SAPK/JNK pathways. In contrast, TNF- α (100 ng/ml) activated phosphorylation of ERK1/2, P38, and SAPK/JNK signaling

regardless of the presence of BMP-2 (Fig. 7A). Densitometric analysis of band intensities showed that phosphorylation of ERK1/2 and SAPK/JNK, but not P38, pathways induced by TNF- α was significantly inhibited by co-treatment with simvastatin (10 μ M; Fig. 7B). The simvastatin effect on inhibiting MAPK activation was not influenced by BMP-2 (Fig. 7B). To determine whether statins regulate the activity of small G-proteins, which are upstream components of MAPK cascades, we next examined the membrane localization of Ras and RhoA. Stimulation with TNF- α (100 ng/ml) potentially increased the level of membrane-bound Ras and RhoA, whereas the effects of simvastatin and BMP-2 were negligible (Fig. 8). Of note, simvastatin (10 μ M) significantly blocked TNF- α -induced Ras and RhoA membrane localization without affecting the total amount of these proteins in C2C12 cells (Fig. 8).

The inhibitory effect of simvastatin (10 μ M) on TNF- α -induced Ras localization was preferentially restored by FPP (5 μ M), while that on TNF- α -induced RhoA localization was reversed by FPP (5 μ M) as well as GGPP (5 μ M; Fig. 9A). FPP also reversed inhibitory effects of simvastatin on ERK1/2 and SAPK/JNK phosphorylation induced by TNF- α (Fig. 9B). GGPP predominantly restored the simvastatin effects on inhibiting TNF- α -induced SAPK/JNK phosphorylation (Fig. 9B). Furthermore, the simvastatin effects on TNF- α suppression of BMP-2-induced Runx2 expression and ALP activity were reversed in the presence of FPP and GGPP (Fig. 9C), suggesting that simvastatin antagonizes TNF- α effects, at least in part, by regulating Ras/Rho-to-MAPK pathway.

To investigate the effect of simvastatin on TNF- α sensitivity, changes in TNF receptor (TNFR) mRNA expression in C2C12 cells were examined in the presence of simvastatin using quantitative real-time PCR. These experiments demonstrated that simvastatin (1 and 10 μ M) significantly decreased expression levels of both TNFR1 and TNFR2 (Fig. 10). TNF- α moderately reduced TNFR1 mRNA expression and had no effect on the expression of TNFR2 (Fig. 10). Thus, simvastatin has dual inhibitory effects on the TNFR signaling, including TNFR expression and the downstream ERK1/2 and SAPK/JNK pathways through Ras/Rho activation (Fig. 11).

Discussion

We recently reported that TNF- α suppresses BMP-2-induced expression of osteoblast markers such as Runx2, osteocalcin, and ALP activity (Mukai *et al.* 2007). Importantly, the inhibition of MAPK pathways, in particular SAPK/JNK, restored TNF- α effects on BMP-induced osteoblast differentiation, suggesting that SAPK/JNK pathway is a key regulator for suppressing BMP signaling caused by TNF- α (Mukai *et al.* 2007). In the present study, we further demonstrated that simvastatin inhibits TNF- α effects in C2C12 cells, leading to maintenance of osteoblast differentiation induced by BMP-2 (Fig. 11). TNF- α suppressed the expression of osteoblastic

Figure 5 Effects of simvastatin and TNF- α on BMP-induced Smad signaling in C2C12 cells. (A and B) After preculture, cells were incubated with simvastatin (10 μ M) in combination with BMP-2 (100 ng/ml) and TNF- α (100 ng/ml). After adding BMP-2 for 1–4 h, cells were lysed and subjected to SDS-PAGE/immunoblot (IB) analysis using antibodies that detect phosphorylated Smad1,5,8 molecules (pSmad1,5,8) and actin. The results shown are representative of those obtained from three independent experiments. (C) The relative integrated density of each protein band (B; the data of 3- and 4-h culture conditions) was digitized by NIH image J 1.34s. Results are shown as mean $\pm$ S.E.M. of data from at least three separate experiments, each performed with triplicate samples. (D) Cells (1×10^5 viable cells) were transiently transfected with Id-1-Luc reporter plasmid (500 ng) and pCMV- β -gal. The cells were pretreated with simvastatin (10 μ M) in combination with BMP-2 (100 ng/ml) for 24 h and then incubated with TNF- α (100 ng/ml). After 24-h culture, the cells were washed with PBS, lysed and the luciferase and β -galactosidase (β -gal) activities were measured by luminometer. The data were expressed as the ratio of luciferase to β -gal activity. Results are shown as mean $\pm$ S.E.M. of data from at least three separate experiments, each performed with triplicate samples. * $P < 0.05$ and ** $P < 0.01$ versus control groups or between the indicated groups.

markers including Runx2 and ALP activity stimulated by BMP-2 in which simvastatin impaired the TNF- α effects on BMP-2-induced osteogenetic process (Fig. 11).

Mundy *et al.* first reported that statins were potent stimulators of bone formation *in vitro* (Mundy *et al.* 1999). In that study, over 30 000 compounds were screened for their ability to stimulate BMP-2 promoter in an immortalized murine osteoblast cell line. The rationale for the approach was that osteoblast differentiation is enhanced by BMPs, whereas other bone growth factors, such as TGFs and fibroblast growth factors (FGFs), stimulate osteoblast proliferation but inhibit osteoblast differentiation. In this regard, Sugiyama *et al.* (2000) also reported that simvastatin, but not pravastatin, induces BMP-2 expression in human osteosarcoma cells. In these experiments, the addition of mevalonate, the downstream metabolite of HMG-CoA reductase, inhibited the statin-mediated activation of BMP-2. Maeda *et al.* (2001) showed stimulatory effects of simvastatin on osteoblastic differentiation in transformed osteoblastic cells and rat bone marrow cells. It also was reported that statins

sequentially induce the expression of stage-dependent markers for osteoblasts, leading to enhancement of osteoblast differentiation (Maeda *et al.* 2004). Thus, there has been accumulating evidence indicating that statins potentially elicit anabolic effects on the osteoblastic differentiation at various stages.

Regarding the functional interaction of statins and cellular BMP system, Hu *et al.* (2006) showed that simvastatin enhances BMP type-II receptor (BMPRII) gene expression in pulmonary artery smooth muscle cells and lung microvascular endothelial cells. Fluvastatin is reported to induce Id-1 expression in human dermal microvascular endothelial cells (Pammer *et al.* 2004). These data provided a convergence of BMP and statin signaling in induction of endothelial Id-1 and implied that these pathways are functionally linked. In the present study, cDNA array analysis of BMP/TGF- β signaling molecules demonstrated enhanced expression of Smad1,2,3 in C2C12 cells treated with simvastatin. Thus, it is likely that the increased Smad expression by simvastatin may be involved in augmenting BMP-induced Smad1,5,8 activation during the process of osteoblast differentiation.

Figure 6 Simvastatin effects on the expression pattern of BMP system molecules in C2C12 cells. (A) Total cellular RNAs were extracted from C2C12 cells treated with TNF- α (10 ng/ml) alone (control group) or simvastatin (10 μ M) in the presence of TNF- α (10 ng/ml) for 24 h (+simvastatin group). Total cellular RNAs (2.0 μ g) were used as templates to generate biotin-16-dUTP-labeled cDNA probes for GEArray[®] membranes (SuperArray Bioscience Corp.) that include human TGF- β and BMP signaling molecules. The cDNA probes were denatured and hybridized with the cDNA array membranes and then the membranes were washed and exposed to X-ray films using chemiluminescent substrate. (B) The spots on the X-ray films were scanned, digitized, and the signal intensities of the spots on the membranes obtained from two separate experiments were analyzed using the GEArray analyzer program (SuperArray Bioscience Corp.) after subtraction of the background levels of pUC18 DNA. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and cyclophilin A (PPIA) are used as a positive control to compare the membranes.

Figure 7 Effects of simvastatin and BMP-2 on TNF- α -induced MAPK phosphorylation in C2C12 cells. (A) After preculture, cells were incubated with simvastatin (10 μ M) in combination with BMP-2 (100 ng/ml) and TNF- α (100 ng/ml). After adding TNF- α for 60 min, cells were lysed and subjected to SDS-PAGE/immunoblot (IB) analysis using anti-phospho-ERK1/2 (pERK1/2), anti-total-ERK1/2 (tERK1/2), anti-phospho-P38 (pP38), anti-total-P38 (tP38), anti-phospho-SAPK/JNK (pSAPK/JNK), and anti-total-SAPK/JNK (tSAPK/JNK) antibodies that detect phosphorylated MAPK signaling. The results shown are representative of those obtained from three independent experiments. (B) The relative integrated density of each protein band was digitized by NIH image J 1.34s. Results are shown as mean $\pm$ S.E.M. of data from at least three separate experiments, each performed with triplicate samples. * P < 0.05 and ** P < 0.01 versus control or between the indicated groups.

Figure 8 Simvastatin effects on TNF- α -induced Ras/Rho membrane localization in C2C12 cells. The cells were incubated with TNF- α (100 ng/ml) or BMP-2 (100 ng/ml) in combination with simvastatin (10 μ M). After adding TNF- α for 60 min, total cellular protein and the membrane fractions were extracted and then subjected to SDS-PAGE/immunoblot (IB) analysis using (A) anti-pan-Ras and (B) anti-RhoA antibodies. The results shown are representative of those obtained from three independent experiments. The relative integrated density of each protein band was digitized by NIH image J 1.34s. Results are shown as mean $\pm$ S.E.M. of data from at least three separate experiments, each performed with triplicate samples. * P <0.05 and ** P <0.01 versus control or between the indicated groups.

TNF- α is a pleiotropic cytokine produced by activated macrophages, which signals through two distinct surface receptors, TNFR1 and TNFR2 (Baud & Karin 2001, Chen & Goeddel 2002). TNF- α has been recognized as a potent stimulator of bone resorption (Mundy 1993) via TNFR1

Figure 9 Effects of farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) on simvastatin actions in C2C12 cells. (A and B) Cells were incubated with TNF- α (100 ng/ml) in combination with simvastatin (10 μ M) and either FPP (5 μ M) or GGPP (5 μ M). After adding TNF- α for 60 min, protein fractions were subjected to SDS-PAGE/immunoblot (IB) analysis for detection of (A) Ras and RhoA (see Fig. 8 legend) and (B) ERK1/2 and SAPK/JNK phosphorylation (see Fig. 7 legend). The results shown are representative of those obtained from three independent experiments. The relative integrated density of each protein band was digitized by NIH image J 1.34s. (C) Cells were pretreated with simvastatin (10 μ M), BMP-2 (100 ng/ml) and either FPP (5 μ M) or GGPP (5 μ M) for 24 h. After treatment with TNF- α (10 ng/ml), cells were subjected to Runx2 mRNA analysis and ALP assay (see Figs 3 and 4 legends). Results are shown as mean $\pm$ S.E.M. of data from at least three separate experiments, each performed with triplicate samples. * P <0.05 and ** P <0.01 versus control groups.

Figure 10 Effects of simvastatin on TNF receptor expression by C2C12 cells. After preculture, the cells were treated with simvastatin (1 and 10 μ M) and TNF- α (30 ng/ml) for 24 h. Total cellular RNA was extracted and subjected to RT reaction. For the quantification of TNF type-1 (A): TNFR1, type-2 receptors (B): TNFR2, and RPL19 mRNA levels, qPCR analysis was performed. The expression levels of target genes were standardized by RPL19 level in each sample. Results are shown as mean $\pm$ S.E.M. of data from at least three separate experiments, each performed with triplicate samples. * $P < 0.05$ and ** $P < 0.01$ versus control groups.

signaling. The cytoplasmic tail of TNFR1 contains a death domain (DD), which is essential for induction of apoptosis; however, this motif is missing in TNFR2 and the function of this receptor is poorly understood (MacEwan 2002). In this study, simvastatin reduced the expression of both TNFR1 and TNFR2 in C2C12 cells (Fig. 11). The expression of TNFR1 and TNFR2 in monocytes has been shown to be modulated by various cytokines including TNF- α , interleukin-10, and macrophage-colony stimulating factor (Takasugi *et al.* 2006). With regard to the effects of TNF- α on osteoblast differentiation, Chen and colleagues suggested a new linkage between BMP and TNF system, showing that BMP-2 and BMP-4 inhibited TNF- α -mediated apoptosis in a NF- κ B-independent manner by inhibition of caspase-8 activation in C2C12 cells (Chen *et al.* 2001). This finding suggests that BMPs not only stimulate osteoblast differentiation but also promote cell survival during osteogenic process.

There has been recent evidence indicating that the effects of TNF- α are primarily associated with activation of MAPK signaling, which subsequently increases the activation of stress-related proteins such as NF- κ B and suppressor of cytokine signaling (SOCS; Wajant *et al.* 2003). Frost *et al.* (2003a) reported that TNF- α stimulated the phosphorylation of SAPK/JNK pathway in C2C12 cells and that the specific inhibition of SAPK/JNK activation, but not other MAPK pathways, prevented TNF- α -induced drop of IGF-I expression. They also showed that SAPK/JNK inhibition blocked TNF-induced IL-6 synthesis in C2C12 cells, suggesting the significance of SAPK/JNK pathway for modulating inflammatory responses in C2C12 cells (Frost *et al.* 2003b). In this regard, we recently showed that TNF- α effects on inhibition of osteogenic process are mediated, at least in part, by the activation of SAPK/JNK pathway and the suppression of an inhibitory Smad6 expression in C2C12 cells (Mukai *et al.* 2007). In the present study, we report that simvastatin potently suppresses TNF- α -induced phosphorylation of ERK1/2 and SAPK/JNK but not P38 pathways. Thus, simvastatin supports BMP-induced osteoblast differentiation through antagonizing TNF- α -to-MAPK pathway as well as augmenting BMP-Smad signaling (Fig. 11).

Inhibition of simvastatin in TNF- α -induced ERK1/2 and SAPK/JNK phosphorylation is likely due to the suppression of small molecular weight G-proteins Ras/Rho in the process of osteoblast differentiation. In our study, TNF- α increased the levels of membrane-bound Ras and RhoA, and simvastatin prevented TNF- α -induced membrane localization of Ras/Rho without affecting the total amount of these proteins in C2C12 cells (Fig. 11). Activation of the mevalonate pathway leads to the production of intermediates, such as FPP and GGPP, which activate Ras and Rho by post-translational modification (Liao & Lauf 2005). Ras/Rho proteins are involved in many of cellular functions such as cell proliferation, differentiation, apoptosis, migration, contraction, and regulation of gene transcription (Liao & Lauf 2005). Activated Ras/Rho proteins are key components in signal-transducing kinase cascades including MAPKs (Shirai *et al.* 2007). The anchoring of these small G-proteins to cell membranes requires prenylation. Ras proteins are farnesylated and Rho proteins are geranylgeranylated (Liao & Lauf 2005). Small G-proteins exist in an inactive GDP-bound cytosolic form and upon cellular activation they exchange GTP and translocate to the active membrane form (Auer *et al.* 2002). By inhibiting this isoprenylation, statins lower membrane levels and activity of Ras/Rho proteins (Auer *et al.* 2002). Based on our results, this process presumably accounts for the present simvastatin effects on antagonizing TNF- α -to-MAPK cascade in C2C12 cells (Fig. 11). In this regard, Fromigué *et al.* (2006) reported that lipophilic statins facilitate membrane RhoA relocation to the cytosol and decrease phosphorylation of ERK1/2 by human osteosarcoma cells. It is further shown that statins induce caspase-dependent apoptosis of osteosarcoma cells through RhoA-MAPK-Bcl-2 pathway independently of BMP-2 signaling (Fromigué *et al.*

Figure 11 A mechanism by which simvastatin antagonizes TNF- α inhibition on BMP-2-induced osteoblastic differentiation. Simvastatin supports BMP-2-induced osteoblast differentiation shown as Runx2 expression and ALP activation through antagonizing TNF- α -to-MAPK pathway and augmenting Smad1,5,8 signaling. SAPK/JNK pathway which is induced by TNF- α is a major regulator for suppressing BMP signaling (Mukai *et al.* 2007). Activation of mevalonate pathway leads to post-translational modification of Ras and Rho such as farnesylation and geranylgeranylation. Activated Ras/Rho proteins are key components for signal-transducing kinase cascades including ERK1/2 and SAPK/JNK. Simvastatin prevents TNF- α -induced membrane localization of Ras/Rho and also reduces the expression of TNFR in C2C12 cells.

2006). Ohnaka *et al.* (2001) have demonstrated stimulatory effects of pitavastatin on the expression of BMP-2 and osteocalcin mRNA in primary cultured human osteoblasts, in which Rho-kinase inhibition was shown as the major mechanism of statin-induced osteoblastic differentiation. Ghosh-Choudhury *et al.* (2007) showed possible signaling crosstalk for the statin-induced osteoblast differentiation. In their study, a lipophilic lovastatin-stimulated Ras activation in osteoblast precursor 2T3 cells, leading to phosphatidylinositol-3-kinase (PI3K) activation, which in turn regulates Akt and ERK phosphorylation to induce BMP-2 expression for osteoblast differentiation (Ghosh-Choudhury *et al.* 2007). Future investigation is necessary to elucidate the underlying mechanism by which statins elicit differential effects on MAPK pathways through Rho/Ras inactivation in the process of osteoblast differentiation.

Taken together with our present data, statins stimulate osteoblast differentiation and matrix mineralization *in vitro*, which implicates clinical applicability of statins for treating osteoporosis. In addition to the effects of statins on endogenous cholesterol levels by inhibiting HMG-CoA reductase, statins also have pleiotropic effects such as anti-inflammatory, anti-proliferative, and anti-thrombotic effects (Rosenson *et al.* 1999, Bellosa *et al.* 2000). Although effects of statins on bone mass and bone turnover remain controversial, increased bone mass, and reduced bone turnover have been observed in patients treated with statins (Garrett & Mundy 2002). The Mundy *et al.* (1999) initial rodent experiments

were remarkable for not only the statin's effects on bone formation but also the anti-resorptive effects since animals given oral simvastatin had a significant reduction in osteoclast number. Additional evidence that statins may have an anti-resorptive effect has been shown by Woo *et al.* (2000) using an *in vitro* assay for osteoclast formation (Takahashi *et al.* 1988), suggesting that statins also exert inhibitory effects on the differentiation of osteoclasts by interfering with the fusion process by which osteoclast precursors develop into multinucleated osteoclast-like cells. Many clinical studies have suggested that statin use is associated with a reduced risk of bone fractures; however, only a modest increase in bone mass and inconsistent effects on bone turnover have been reported to date (Bauer 2003, Jadhav & Jain 2006). Given that <5% of an oral dose statin reaches the systemic circulation (Bellosa *et al.* 2000), osteoblasts and osteoclasts are exposed to very low concentrations of statin with usual oral regimens. Specific statins with high affinity to bone tissues would be useful for prevention or treatment of osteoporosis due to inflammatory cytokines associated with rheumatoid arthritis.

Acknowledgements

We thank Dr R Kelly Moore for helpful discussion and critical reading of the manuscript. We also thank Drs Tetsuro Watabe and Kohei Miyazono for providing Id-1-Luc plasmid. This work was supported in part by Grants-in-Aid for

Scientific Research, Life Science Foundation of Japan, Takeda Science Foundation, Sakakibara Memorial Research Grant, The Kawasaki Foundation for Medical Science and Medical Welfare, The Ichiro Kanahara Foundation, Kato Memorial Bioscience Foundation, Terumo Lifescience Foundation, and Japan Rheumatism Foundation. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received in final form 15 December 2007

Accepted 3 January 2008

Made available online as an Accepted Preprint
3 January 2008