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Salicylideneamino-2-thiophenol Enhances Osteogenic Differentiation Through the Activation of MAPK Pathways in Multipotent Bone Marrow Stem Cell

Hyung Keun Kim,[1,2] Kyung Soon Park,[1] Jun Sik Lee,[3] Ji Hyun Kim,[1,2] Dae Sung Park,[1,2] Jung-Woog Shin,[4*] and Taek Rim Yoon[1,2**]

[1]*Department of Orthopaedic Surgery, Center for Joint Disease, Chonnam National University, Hwasun Hospital, Hwasun, Korea*

[2]*Heart Research Center, Chonnam National University Hospital, Gwangju 501-757, Korea*

[3]*Department of Biology, College of Natural Science, Chosun University, Gwangju 501-759, Korea*

[4]*FIRST Research Team/Institute of Aged Life Redesign/Cardiovascular and Metabolic Disease Center, Inje University, Gimhae, Korea*

ABSTRACT

Osteoporosis is a reduction in skeletal mass due to an imbalance between bone formation and bone resorption. Therefore, the identification of specific stimulators of bone formation is of therapeutic significance in the treatment of osteoporosis. Salicylideneamino-2-thiophenol (Sal-2) consists of two benzene rings, has been reported to possess antioxidant activity, and is an effective remedy for fever and rheumatic diseases. However, until now the effects of osteoblastic bone formation by Sal-2 were unknown. In this study, we investigated the effects of Sal-2 on osteogenic differentiation of multipotent bone marrow stromal stem cells by alizarin red S staining for osteogenic differentiation, RT-PCR and western blot for alkaline phosphatase (ALP) activity and signaling pathways, FACS analysis and immunofluorescence staining for CD44 and CD51 expression, calcium assays, and immunofluorescence staining for signaling pathways. We found that Sal-2 enhanced the osteogenic differentiation of multipotent bone marrow stromal stem cells. Sal-2 treatment induced the expression and activity of ALP, and enhanced the levels of CD44 and CD51 expression as well as Ca^{2+} content, in multipotent bone marrow stromal stem cells. Moreover, we found that Sal-2-induced osteogenic differentiation and expression of osteogenesis-related molecules involve the activation of the MAPK and nuclear factor-κB pathways. Our findings provide insight into both the mechanism and effects of Sal-2 on osteogenic differentiation and demonstrate that Sal-2 may be a beneficial adjuvant in stimulating bone formation in osteoporotic diseases. J. Cell. Biochem. 113: 1833–1841, 2012.
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KEY WORDS: SALICYLIDENEAMINO-2-THIOPHENOL; MULTIPOTENT BONE MARROW STROMAL STEM CELLS; OSTEOGENIC DIFFERENTIATION; MAPK

Bone mass is the end product of a complex process characterized by a balance between bone resorption by osteoclasts and bone formation by osteoblasts [Tsai et al., 2004]. Bone loss is due to excessive osteoclastic bone resorption relative to osteoblastic bone formation and leads to osteoporosis [Huang et al., 2007]. Therefore, both specific suppressors of bone resorption and stimulators of bone formation are of therapeutic significance in the treatment of osteoporosis.

Mesenchymal stem cells (MSCs) are capable of differentiating into a variety of cell types, including osteoblasts, chondrocytes, and adipocytes [Lavery et al., 2009]. MSCs derived from bone marrow have been used to repair skeletal bone and hard tissue. Therefore, MSCs constitute an interesting target for use in cell therapy, including bone grafts and allograft implant. In previous studies, we and another group used multipotent bone marrow stromal stem cells as an MSC cell line. These cells were cloned from mouse bone

Conflict of interest: None.

Hyung Keun Kim and Kyung Soon Park contributed equally to the work.

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*Correspondence to: Jung-Woog Shin, Inje University, Gimhae, 621-749, Korea. E-mail: sjw@bme.inje.ac.kr

**Correspondence to: Taek Rim Yoon, Department of Orthopaedic Surgery, Center for Joint Disease of Chonnam National University Hospital, Gwangju, 501-757, Korea. E-mail: tryoon@jnu.ac.kr

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marrow stromal stem cells, which can be differentiated into the osteogenic lineage when cultured in osteogenic differentiation medium (ODM) containing dexamethasone, ascorbic acid, and β-glycerolphosphate [Dahir et al., 2000; Kim et al., 2009]. Furthermore, we have reported that mevinolin-treated multipotent bone marrow stromal cells undergo osteogenic differentiation through the enhancement of osteogenic genes [Kim et al., 2009].

Salicylideneamino-2-thiophenol (Sal-2) consists of two benzene rings, like other well-known natural antioxidants, and has been used as an effective remedy for fever and rheumatic diseases. In addition, Sal-2 has a thiol group, which can modulate inflammatory responses by reducing oxidative stress [Santangelo, 2003]. Sodium salicylate has anti-inflammatory effects via cylcooxygenase-2 inhibition [Amann et al., 2001] and the suppression of NF-κB signaling pathways [Kwon and Chae, 2003]. Moreover, we have reported that Sal-2 inhibited *tert*-butyl hydroperoxide-induced inflammatory mediator genes and the activation of the related signaling pathway [Chung et al., 2008]. However, the effect of Sal-2 on osteoblastic bone formation has not yet been investigated.

In the present study, we determined if Sal-2 regulates the differentiation and function of osteoblasts by utilizing mouse multipotent bone marrow stromal stem cells. Our results show that Sal-2 treatment results in an increase in osteogenic differentiation in multipotent bone marrow stromal stem cells and enhances the expression of osteogenic genes and proteins, including alkaline phosphatase (ALP) and Runx2. Moreover, osteogenic differentiation in multipotent bone marrow stromal stem cells by Sal-2 is mediated by MAPK and NF-κB signaling pathways.

MATERIALS AND METHODS

OSTEOGENIC DIFFERENTIATION

The multipotent bone marrow stromal stem cells (D1 cells) were cultured as previously described [Dahir et al., 2000]. D1 cells were purchased from the ATCC (Manassas, VA) and maintained in DMEM containing 10% FBS (GIBCO BRL) and antibiotics (GIBCO). Cells were seeded at 1×10^4 cells/well and maintained in culture for 3 days in a humidified 5% CO_2 atmosphere at 37°C. Experiments were performed after cells had reached about 80% confluence. To induce osteogenic differentiation, culture media were changed at 3 days to ODM [DMEM supplemented with 50 μg/ml ascorbic acid, 10^{-8} M dexamethasone, and 10 mM β-glycerolphosphate (all from Sigma-Aldrich, St. Louis, MO)]. After another 3 days, one group was cultured in only ODM, while another group was cultured in ODM plus Sal-2 (0.01, 0.1, or 1 μg/ml). Cells were then analyzed 24 or 48 h later.

ALIZARIN RED S STAINING

Calcium deposits on cells were quantified as described previously [Chen et al., 2005]. Briefly, cell cultures were washed twice with distilled water, fixed for 1 h in ice-cold 70% ethanol, and rinsed twice with deionized water. Cultures were stained for 10 min with alizarin red S solution, and excess dye was removed gently using running water. Calcium deposits, which appeared bright red, were identified by light microscopy and photographed. Osteogenic differentiation was quantified by determining densities and areas of alizarin red S staining using an image analysis program (Multi Gauge V3.0, Fujifilm, Japan).

ALP ACTIVITY ASSAYS

Specific ALP activities were assayed using the LabAssay™ ALP assay kit (Wako, Osaka, Japan). After rinsing the monolayers with Ringer solution, the cells were lysed and the cellular material was transferred into a buffer containing 10 mM Tris HCL (pH 7.5), 0.5 mM $MgCl_2$, and 0.1% Triton X-100. The cellular material was homogenized by two freeze-thaw cycles. ALP activity was determined with *p*-nitrophenylphosphate as the substrate. Samples were incubated at 37°C for 30 min on a bench shaker with the substrate solution of *p*-nitrophenylphosphate (4.34 mM), 100 mM glycine (pH 10.3), and 1 mM $MgCl_2$. The enzymatic reaction was stopped by adding 1 M NaOH.

Optical densities of the *p*-nitrophenol produced were read at 405 nm using an Infinite M200 microplate reader (Tecan Austria GmbH). ALP activities were normalized to total protein content, which was determined using a Bradford assay kit (Bio-Rad, Hercules, CA).

RT-PCR AND REAL-TIME PCR ANALYSIS

To assess the effects of Sal-2 on the transcription of genes encoding ALP (5′-ACA CCT TGA CTG TGG TTA CTG CTG A-3′; 5′-CCT TGT AGC CAG GCC CGT TA-3′), osteocalcin (5′-GAG GGC AAT AAG GTA GTG AAC AGA-3′; 5′-AAG CCA TAC TGG TCT GAT AGC TCG-3′), Runx2 (5′-ACA AAC AAC CAC AGA ACC ACA AGT-3′; 5′-GTC TCG GTG GCT GGT AGT GA-3′), and the housekeeping enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 5′-AAA TGG TGA AGG TCG GTG TG-3′; 5′-TGA AGG GGT CGT TGA TGG-3′), real-time RT-PCR was performed. Briefly, D1 cells grown to 70% confluence on plates with/without Sal-2 were homogenized using TRIzol reagent (Life Technologies, Carlsbad, CA). Total RNA was then isolated, and 0.5 μg RNA aliquots were reverse transcribed in 20 μl buffer containing 5× AMV reverse transcriptase, 2.5 μM poly dT, 1 mM each of dATP, dCTP, dGTP, and dTTP, 20 U of RNAse inhibitor, and 20 U of AMV RT. Reverse transcription was performed using the following conditions in a GeneAmp PCR System 2700 (Applied Biosystems, Foster City, CA): Initial incubation at room temperature for 10 min, followed by incubation at 42°C for 15 min, 97°C for 5 min, and at 5°C for 5 min. Aliquots of cDNA were amplified in AccuPower® GreenStar qPCR premix (Bioneer Co., Daejeon, Korea) using an ExiCycler™ 96 Real-time Quantitative Thermal Block (Bioneer Co.).

WESTERN BLOT ANALYSIS

Cells were harvested, washed twice with ice-cold PBS, and lysed in RIPA buffer (Sigma-Aldrich) supplemented with a protease inhibitor cocktail (Sigma-Aldrich) for 1 h on ice with vortexing every 10 min. Lysates were centrifuged at 8,000*g* for 20 min to remove insoluble material. Protein concentration in supernatants was determined using a Bradford assay kit using BSA as a standard. Equal amounts of protein were separated on 10 or 12% SDS–PAGE gels, and blots were subsequently transferred to nitrocellulose membranes (Hybond C, Amersham Life Sciences, Uppsala, Sweden). Membranes were treated with primary antibodies against ALP, osteocalcin, Runx2,

phospho-p38 MAPK, p38 MAPK, phospho-ERK1/2, ERK1/2, phospho-p65, Topo I, and β-actin (Santa Cruz Biotechnology Inc., Santa Cruz, CA), and secondary antibodies were used at 1:500–1:1,000. Proteins were detected by an enhanced chemiluminescence reagent using a commercial kit (Amersham Life Sciences). Protein expression was quantified by determining blot densities using an LAS-3000 system (Fujifilm, Tokyo, Japan).

IMMUNOFLUORESCENCE STAINING

D1 cells grown on coverslips were fixed with 4% paraformaldehyde in PBS for 15 min, permeabilized with 0.1% Triton X-100 for 15 min, and then blocked with 5% BSA in PBS for 30 min. Coverslips were then incubated with primary antibodies against mouse CD44, CD51, phospho-p38 MAPK, phospho-ERK1/2, or phospho-p65 (eBioscience, San Diego, CA) at a dilution of 1:100. Secondary antibodies were used at 1:200 dilutions. Both primary and secondary antibody incubations were performed at room temperature for 1 h. Cells were then washed with PBS and mounted in 70% glycerol. Photomicrographs were obtained using an Olympus BX50 fluorescence microscope (Olympus, Tokyo, Japan). Nuclei were stained using DAPI solution.

CALCIUM ASSAYS

The measurement of calcium content was performed as previously reported [Hernandez et al., 2007]. The calcium was assayed using the QuantiChrom calcium assay kit (Gentaur, Kampenhout, Belgium) according to the manufacturer's recommendations; this assay is based on the fact that the phenolsulfonephthalein dye in the kit forms a very stable blue-colored complex when it binds specifically with free calcium. The intensity of the color, measured at 612 nm using an Infinite M200 microplate reader, is directly proportional to the calcium concentration in the sample.

FLOW CYTOMETRIC ANALYSIS

D1 cells (0.5×10^5) were incubated in staining buffer (PBS containing 0.5% FBS and 0.1% sodium azide) containing anti-CD44 and anti-CD51 (eBioscience) for 30 min on ice. Cells were stained with the appropriate isotype-matched Ig and used as negative controls. After staining, cells were fixed with 2% paraformaldehyde and analyzed using a FACSCalibur equipped with CellQuest software (BD Biosciences).

STATISTICAL ANALYSIS

Results are presented as mean ± standard deviation (SD). Data were analyzed by one-way analysis of variation (ANOVA), followed by Duncan's post hoc test using SPSS version 11.0 (SPSS, Inc., Chicago, IL). Throughout the figures and legends the following terminology has been used to denote statistical significance: $^{**}P < 0.01$, $^{*}P < 0.05$.

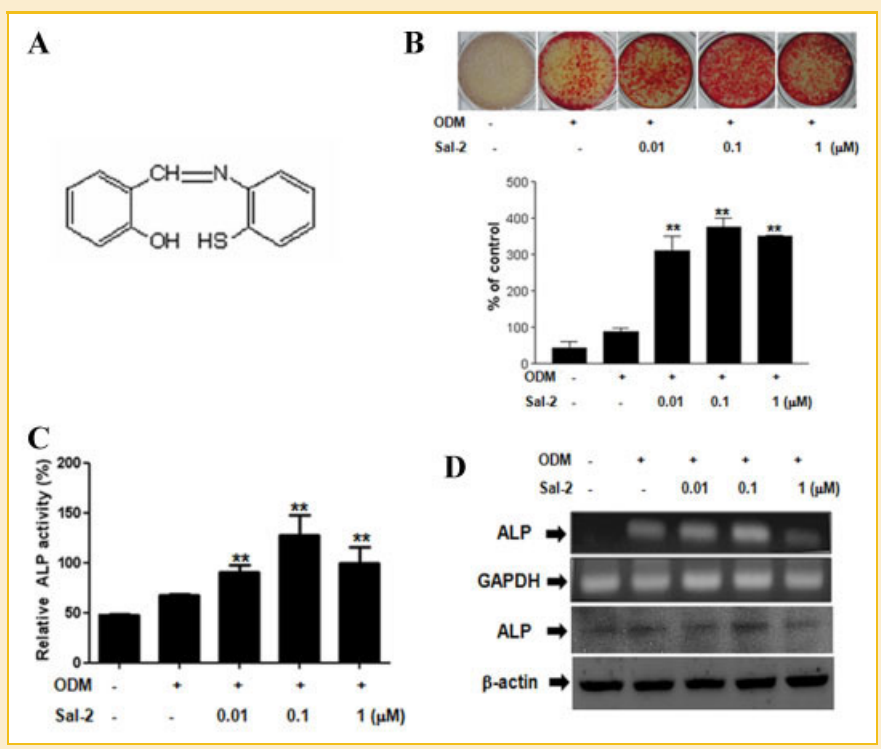

Fig. 1. Effect of Sal-2 on osteogenic differentiation, ALP expression, and ALP activity in D1 cells. The treatment of Sal-2 (A) during the first 3 days of differentiation resulted in a significant increase in osteogenic differentiation in D1 cells. D1 cells were treated with various concentrations (0.01–1 μg/ml) of Sal-2 during the initial phase of osteogenic differentiation (B). ALP activity was assayed by the production of *p*-nitrophenol from *p*-nitrophenyl phosphate (C). The expression of ALP was measured by RT-PCR and western blot (D). Data are mean ± SD of four independent experiments. $^{**}P < 0.01$ for a comparison with ODM medium control.

RESULTS

SAL-2 INDUCES OSTEOGENIC DIFFERENTIATION

To determine if Sal-2 (chemical structure shown in Fig. 1A) affects osteogenic differentiation, the D1 cells were treated with various concentrations (0.01, 0.1, or 1 μg/ml) of Sal-2 during the initial phase of osteogenic differentiation. The treatment of Sal-2 during the initial phase of differentiation resulted in a significant increase in osteogenic differentiation in multipotent bone marrow stromal stem cells (Fig. 1B). ALP is a marker of osteoblasts in the proliferative stage [Macdonald et al., 2010; Pilz et al., 2011]; thus, we then determined if treatment with Sal-2 is sufficient to enhance ALP activity and expression. D1 cells treated with various concentrations of Sal-2 showed increased ALP activity and expression compared with ODM control (Fig. 1C). Moreover, Sal-2-treated multipotent bone marrow stromal stem cells enhanced the gene expression and protein levels of ALP (Fig. 1D).

MSCs such as multipotent bone marrow stromal stem cells undergo mitotic clonal expansion upon exposure to differentiation media [Kim et al., 2006]. We assessed the effects of ODM on cell proliferation. We found that ODM had no effects on cell proliferation but that Sal-2 treatment increased cell proliferation (data not shown).

SAL-2 INDUCES Runx2 AND OSTEOCALCIN AND INCREASES CA^{2+} CONTENT

Ca^{2+} ions play a fundamental role in many cellular processes, and the extracellular concentration of Ca^{2+} is important to maintain bone mass and density [Winzenberg et al., 2006a; Winzenberg et al., 2006b]. To determine if Sal-2 affects calcium content during osteogenic differentiation, we measured the calcium content of cell cultures after Sal-2 treatment. Sal-2 treatment in D1 cells induced an increase in Ca^{2+} content (Fig. 2A). In order to understand the action of Sal-2 on D1 cells at the molecular level, we measured the effects of Sal-2 on the expression of the genes involved in osteogenesis, including osteocalcin and Runx2. RT-PCR and western blot analysis showed that both osteocalcin and Runx2 expression increased during osteogenic differentiation in Sal-2-treated D1 cells as compared with multipotent D1 cells in ODM only (Fig. 2B,C). Furthermore, we found that the optimal dosage of Sal-2 for osteogenic differentiation is 0.1 μg/ml in D1 cells.

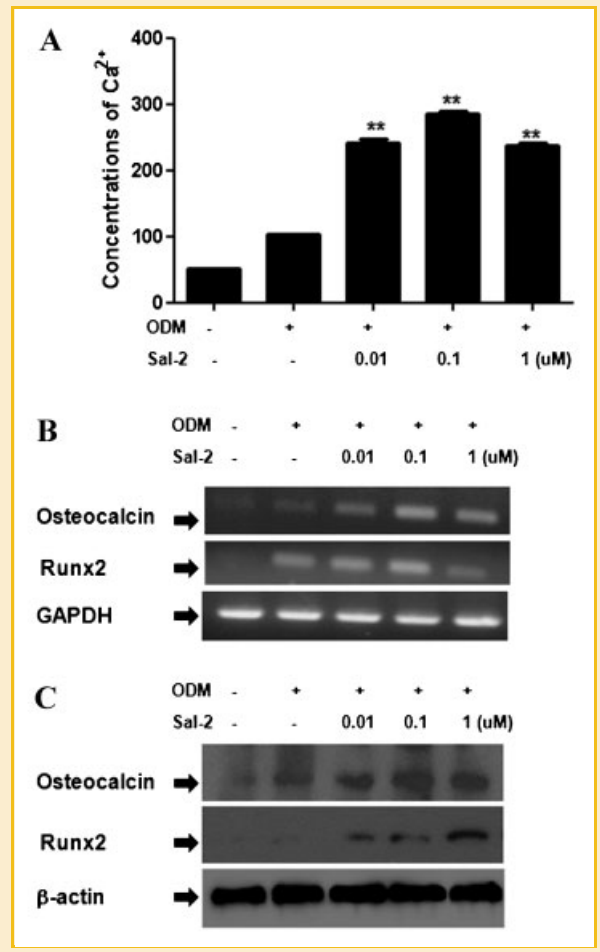

Fig. 2. Effect of Sal-2 on Ca^{2+} content and the expression of osteocalcin and Runx2 in D1 cells. Cells were treated with various concentrations (0.01–1 μg/ml) of Sal-2 during osteogenic differentiation. Then, Ca^{2+} content was measured (A). Total RNA was isolated from control, ODM only, or Sal-2-treated groups (B). Lysates were prepared from the Sal-2-treated groups and were subjected to western blot analysis using antibodies against osteocalcin and Runx2 (C). Data are mean ± SD of four independent experiments. $^{**}P < 0.01$ for a comparison with ODM medium control.

SAL-2 INDUCES CD44 AND CD51 EXPRESSION

CD44 and CD51 are expressed in a variety of cell types [Wang et al., 2004]. Previously, several reports have shown that CD44 and CD51 are expressed during osteogenic differentiation and act as biomarkers for osteogenesis [Zohar et al., 1998; Berner et al., 2004; Kim et al., 2010]. We next investigated the effect of Sal-2 on CD44 and CD51 expression in D1 cells. CD44 and CD51 expression was measured by flow cytometry and fluorescence microscopy. As shown in Figure 3A,B, treatment with Sal-2 induced the expression of CD44 and CD51 in D1 cells.

SAL-2 INDUCES OSTEOGENESIS VIA THE ACTIVATION OF MAPK AND NF-κB PATHWAYS

Mitogen-activated protein kinases (MAPKs), including p38 MAPK and ERK1/2, and NF-κB (p65) have been implicated in the osteogenic differentiation pathway [Hamidouche et al., 2009; Greenblatt et al., 2010]. To investigate the mechanism underlying the induction of osteogenic differentiation by Sal-2, we have determined the effects of Sal-2 treatment on these signaling proteins. As shown in Figure 4A, Sal-2 significantly induced the phosphorylation of p38 MAPK and ERK1/2 at 30 min. Next, to investigate the direct correlation of Sal-2 action with the activation of MAPK signaling pathways, we determined the effects of MAPK-specific inhibitors on the Sal-2-induced activation of MAPK signal pathway in D1 cells. As shown in Figure 4B, pretreatment with the inhibitors SB203580 and U0126 inhibited the Sal-2-induced activation of p38 MAPK and ERK1/2 in D1 cells. We confirmed that Sal-2 induced the activation of MAPK signal pathways by immunofluorescence staining (Fig. 4C). Next, we investigated the activation of the NF-κB signaling pathway by western blot of nuclear protein extract and immunofluorescence staining. As shown

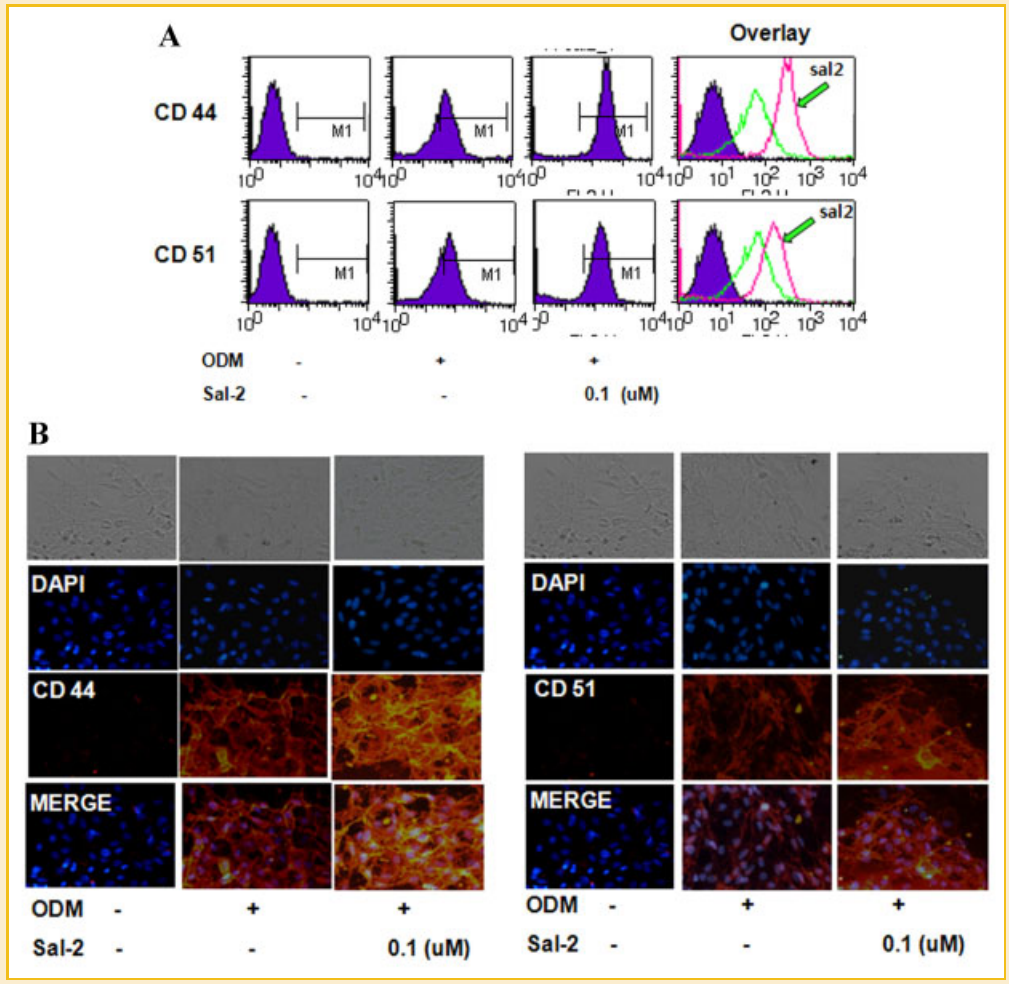

Fig. 3. Effect of Sal-2 on the expression of CD44 and CD51 in D1 cells. D1 cells were treated with Sal-2 (0.1 μg/ml) or ODM only and CD44 and CD51 expression was measured by flow cytometry (A) and fluorescence microscopy (B). Data are representative of three independent experiments. Data are representative of four independent experiments. Magnification 20×.

in Figure 4D, Sal-2 significantly induced the activation and translocation of NF-κB in D1 cells.

MAPK PATHWAYS ARE ESSENTIAL FOR OSTEOGENESIS BY SAL-2

Based on our finding that Sal-2 activates the MAPK signaling pathway, which has been implicated in osteogenic differentiation, we sought to determine if Sal-2 achieves its osteogenic function through this pathway. To do this, we determined the effects of MAPK inhibition on CD44 and CD51 expression during Sal-2-induced osteogenic differentiation. As shown in Figure 5A, CD44 and CD51 expression was increased by Sal-2, but pretreatment with MAPK inhibitors decreased the Sal-2 induction CD44 and CD51 expression. Furthermore, we assessed ALP activity and Ca^{2+} content with inhibition of MAPK during Sal-2-induced osteogenic differentiation. We found that, when MAPK pathways were specifically inhibited, Sal-2-induced ALP activity and Ca^{2+} content were decreased. These data suggest that osteogenic differentiation induced by Sal-2 requires the activation of the MAPK signaling pathway in D1 cells (Fig. 5B). Interestingly, in case of Ca^{2+} content showed a high potent of the activation of p38 MAPK during Sal-2-induced Ca^{2+} content as compared with ERK1/2 inhibitors treated groups (Fig. 5C).

THE ACTIVATION OF P38 MAPK IS CRITICAL FOR SAL-2-INDUCED OSTEOGENIC DIFFERENTIATION

As Ca^{2+} content is an important for osteogenic differentiation and bone density [Muller et al., 2008], we assessed the effects of p38 MAPK inhibition on Sal-2-induced osteogenic differentiation in D1 cells. As shown in Figure 6, osteogenic differentiation analysis showed that SB203580 significantly inhibited osteogenic differentiation induced by Sal-2 compared with the U0126-treated group. These data suggest that p38 MAPK is essential for osteogenic differentiation induced by Sal-2 treatment.

DISCUSSION

Because new bone formation is primarily a function of the osteoblast, agents that act by either inducing differentiation of

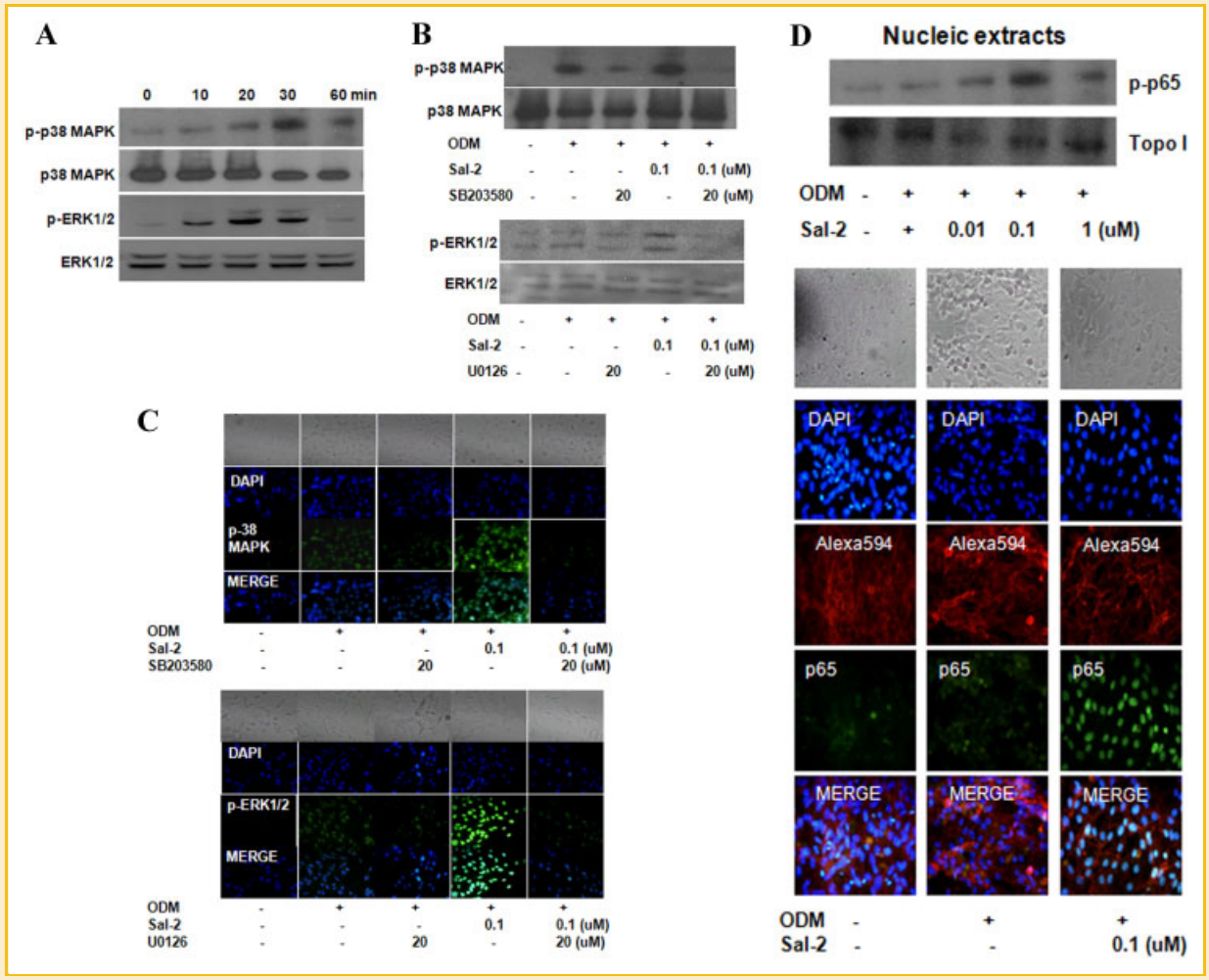

Fig. 4. Effect of Sal-2 on the activation of MAPK and NF-κB in D1 cells. D1 cells were cultured in ODM with 0.1 μg/ml Sal-2 in various time courses. The cell lysates were prepared and blotted with anti-phospho-p38 MAPK and anti-phospho-ERK1/2 antibodies. The bound antibodies were visualized using biotinylated goat anti-mouse IgG (A). Cells were cultured in ODM in the absence or presence of 0.1 μg/ml Sal-2 and with or without 20 μM of MAPK-specific inhibitors (SB203580, U0126) (B). Photographs of phospho-p38 MAPK and phospho-ERK1/2 in D1 cells cultured in ODM in the presence or absence of Sal-2 and inhibitors as determined by fluorescence microscopy (C). Data are representative of four independent experiments. Magnification 20×.

the osteoblasts or increasing the proliferation of cells of the osteoblastic lineage can enhance bone formation. Therefore, the screening of chemicals that enhance osteogenic differentiation may serve to expand the number of adjuvants for clinical applications of bone engineering using MSCs. To investigate if Sal-2 induces osteogenic differentiation, we used D1 cells, which are capable of differentiating into either the osteoblast or the adipocyte lineages [Cui et al., 1997; Dahir et al., 2000; Li et al., 2003; Hung et al., 2008; Kim et al., 2009]. We, along with other groups, reported that D1 cells can differentiate into the osteogenic lineage when cultured in ODM containing dexamethasone, ascorbic acid, and β-glycerolphosphate [Cui et al., 1997; Dahir et al., 2000; Kim et al., 2009].

In this study, we found that Sal-2 induced osteogenic differentiation and elucidated essential signaling pathways in D1 cells. Moreover, Sal-2 induced the expression of surface molecules, including CD44 and CD51 (Fig. 3A,B). To determine if Sal-2 has effects on osteogenic differentiation, we analyzed the expression of ALP and its activity. ALP expression and activity are considered markers of osteogenic differentiation. It also has been reported that early progenitor cells do not express osteoblast markers such as ALP, Runx2, and osteocalcin. Those markers are expressed when the cell has differentiated into a mature osteoblast [Kim et al., 2009]. In the present study, we assessed the effect of Sal-2 on both the expression and activity of ALP. ALP expression and activity were dose-dependently increased in the Sal-2-treated group compared with the ODM only group (Fig. 1C). Runx2 is a key transcription factor associated with osteoblast differentiation. It is critical for osteoblastic differentiation and skeletal morphogenesis [Li et al., 2008]. Osteocalcin is a γ-carboxyglutamic acid-containing, noncollagenous protein found in bone and secreted by osteoblasts, which plays a role in metabolic regulation and bone-building [Lumachi et al., 2009; Kenanidis et al., 2011]. Our RT-PCR and western blot data demonstrate that treatment with Sal-2 induced an increase in osteogenic differentiation-related markers, such as Runx2 and osteocalcin,

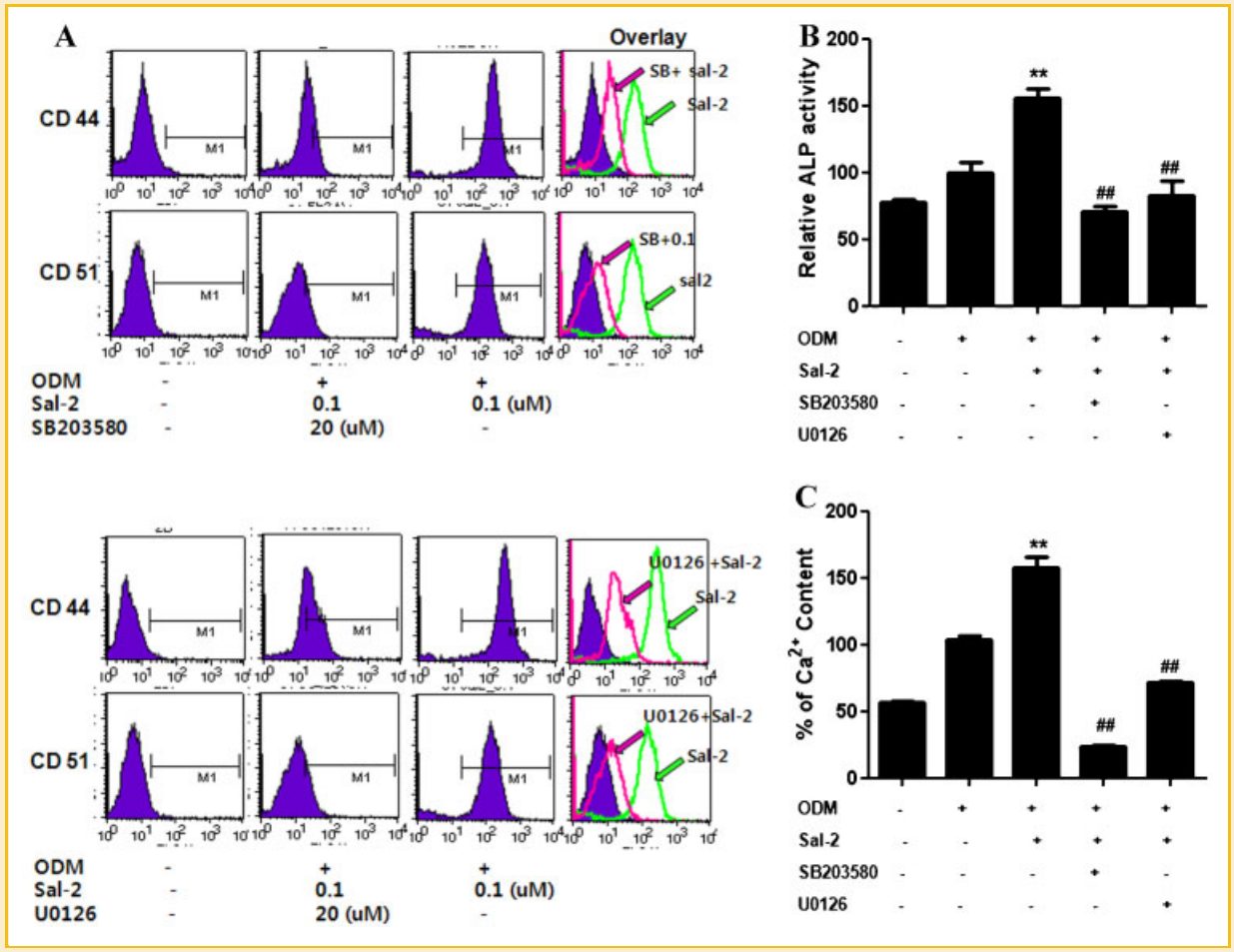

Fig. 5. Effect of the inhibition of MAPK on Sal-2-induced CD44, CD51, ALP activity, and Ca^{2+} content. Cells were cultured in ODM in the absence or presence of 0.1 μg/ml Sal-2 and with or without 20 μM of MAPK-specific inhibitors (SB203580, U0126). CD44 and CD51 expression was measured by flow cytometry (A). ALP activity was assayed the production of *p*-nitrophenol from *p*-nitrophenyl phosphate (B). Ca^{2+} content was measured (C). Data are mean ± SD of four independent experiments. $^{**}P < 0.01$ for a comparison with ODM medium control. $^{\#\#}P < 0.01$ for a comparison with Sal-2-treated group.

as well as increased Ca^{2+} content (Fig. 2). However, in assessing treatment with various concentrations of Sal-2 in osteogenic differentiation and ALP expression, we found that 1 μg/ml of Sal-2 diminished the effect of inducible osteogenic differentiation and ALP activity. To this end, we determined the cytotoxicity of various concentrations of Sal-2. Although 1 μg/ml of Sal-2 seems to have diminished effect, this is not due to cytotoxicity of Sal-2 according to the cytotoxicity data (data not shown). These data suggest the optimal concentration of Sal-2 for osteoblast differentiation is 0.1 μg/ml in D1 cells.

ERK1/2 and p38 MAPK are important for differentiation and proliferation of osteoblasts. In particular, p38 MAPK is involved in the regulation of ALP expression during osteoblastic differentiation. Our data show that Sal-2 treatment induces the activation of p38 MAPK and ERK1/2 in D1 cells. Moreover, it has been reported that insulin stimulates osteoblast proliferation and differentiation via the NF-κB pathway [Wang et al., 2009]. In addition, we determined if Sal-2 induces the activation of NF-κB pathway. Interestingly, we found that treatment with Sal-2 increased the activation of the p65 NF-κB subunit in D1 cells. Taken together, these data indicate that Sal-2-induced osteogenic differentiation involves the activation of the p38 MAPK, ERK1/2, and NF-κB pathways. Activation of these signaling pathways by Sal-2 was confirmed by their specific inhibitors (Fig. 4A–D).

CD44 acts as a receptor for hyaluronic acid, which is involved in adhesion to the extracellular matrix, cellular proliferation, migration, and differentiation [Kim et al., 2009]. It has been reported that CD51 is a ubiquitous receptor that interacts with several ligands, such as osteopontin and matrix metalloproteinase-2 (MMP-2). Our results show that treatment with Sal-2 induced expression of both CD44 and CD51. Furthermore, we found that the expression of CD44 and CD51 was decreased by p38 MAPK and ERK1/2 inhibitors (SB203580 and U0126). Taken together, our results indicate that osteogenic differentiation induced by Sal-2 is significantly inhibited by inhibiting p38 MAPK and ERK1/2 (Figs. 5 and 6). Moreover, osteogenic differentiation induced by Sal-2 is more dependent on

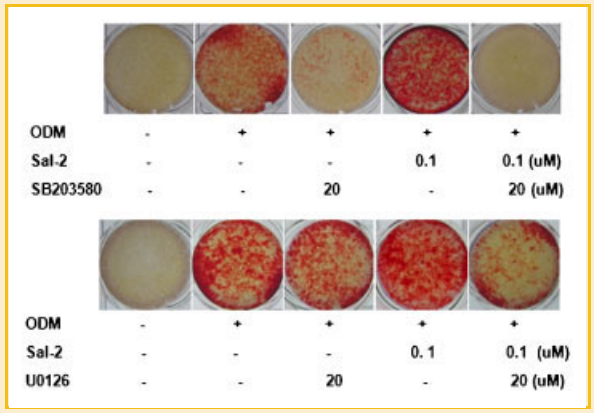

Fig. 6. Effect of MAPK inhibition on Sal-2-induced osteogenic differentiation. D1 cells were treated with various concentrations (0.01–1 μg/ml) of Sal-2 and with or without 20 μM of MAPK-specific inhibitors (SB203580, U0126) during the initial phase of osteogenic differentiation. Cultures were fixed with 70% ethanol and mineralized nodule formation was assessed by alizarin red-S staining. Data are representative of four independent experiments.

p38 MAPK activation relative to ERK1/2 activation. In the present study, our results suggest that Sal-2 treatment enhances osteogenic differentiation by increasing osteogenic surface markers through the MAPK pathway.

In conclusion, the present study demonstrates that Sal-2 stimulates osteoblast differentiation through an increase in Ca^{2+} content and upregulation of ALP, osteocalcin, and Runx2. Moreover, Sal-2 induction of these osteogenic markers during osteogenic differentiation is dependent on MAPK. Therefore, these results suggest that Sal-2 may be a beneficial adjuvant to stimulate bone formation in osteoporotic diseases.

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