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NOD2 Mediates Odontoblast Differentiation and RANKL Expression

ABSTRACT

The precise regulation of odontoblast differentiation and osteoclastogenic cytokine expression in human dental pulp cells (HDPCs) is crucial for the pathology of bacteria-related pulpitis. Although the up-regulation of nucleotide-binding oligomerization domain-containing protein 2 (NOD2) has been reported in inflamed human dental pulps, the role of NOD2 in the differentiation of HDPCs remains unclear. Here, we show the involvement of NOD2 in odontoblast differentiation together with osteoclastogenic cytokine expression in HDPCs. Treatment with muramyl dipeptide (MDP), a known NOD2-agonist, significantly inhibited odontoblast differentiation of HDPCs, as revealed by reduced ALP activity, osteoblast/odontoblast marker expression, and mineralized nodule formation. Importantly, the forced down-regulation of NOD2 by small interfering RNA (siRNA) recovered MDP-down-regulated odontoblast differentiation. MDP-elicited suppression of odontoblast differentiation resulted from the increased expression of MKP-1 protein and the subsequent decline of MAPKs phosphorylation, which is a prerequisite for odontoblast differentiation. Furthermore, we found that MDP treatment elevated the expression of osteoclastogenic cytokines in HDPCs, which was also reversed by NOD2 silencing. Analysis of these data, taken together, suggests that the regulation of NOD2 expression upon MDP challenge might serve as an intrinsic mechanism that underlies the hindered dentin formation and accelerated dentin resorption in bacterial infection-mediated pulpitis.

Abbreviations: HDPCs, human dental pulp cells; NOD2, nucleotide-binding oligomerization domain-containing protein 2; MDP, muramyl dipeptide; siRNA, small interfering RNA; MAPKs, mitogen-activated protein kinases; MKP-1, MAPK phosphatase-1; BMMs, bone-marrow-derived macrophages; and CM, conditioned medium.

INTRODUCTION

Pulpitis is characterized as the immune response that is primarily triggered by the invasion of caries-related bacteria into dentin tissue and pulp (Adachi *et al.*, 2007). With pulpitis development, increased expression of pro-inflammatory mediators is found in inflamed pulp, especially in human dental pulp cells (HDPCs) (Nakanishi *et al.*, 1995). Pro-inflammatory cytokines are produced by the immune system in response to the recognition of pathogen-related molecular patterns (PAMPs), such as lipopolysaccharide (LPS), peptidoglycan, and nucleic acid variants, by the immune system.

Recently, it has been reported that HDPCs express TLR2 and TLR4 and therefore retain the ability to participate in the innate immune response (Mutoh *et al.*, 2007). Similar to TLRs, nucleotide-binding oligomerization domain-containing protein 1 (NOD1) and NOD2 are intracellular pattern-recognition receptors, which recognize a specific molecular structure called muramyl dipeptide (MDP) in Gram-positive/negative bacteria (Girardin *et al.*, 2003). Stimulation of NODs induces the expression of inflammatory cytokines *via* the activation of nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) signaling (Inohara and Nuñez, 2003). Among the 2 isoforms, NOD2 is expressed in healthy dental pulp (Hirao *et al.*, 2009) and is known to be up-regulated in inflamed pulps challenged by cariogenic bacteria (Keller *et al.*, 2011).

Osteoblasts express NOD1 and NOD2 and therefore participate in the release of inflammatory cytokines and support osteoclastogenesis by providing macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor-κB ligand (RANKL) (Marriott *et al.*, 2005). *Escherichia coli* LPS has been shown to interact with TLR4 on the surfaces of osteoblasts and up-regulates the expression of RANKL and down-regulates that of osteoprotegerin (OPG), a decoy receptor for RANKL (Suda *et al.*, 2004). More importantly, NOD2-mediated signals are involved in MDP-induced RANKL expression in osteoblasts (Yang *et al.*, 2005).

Recently, it was reported that the expression of cyclo-oxygenase-2 (COX2) and prostaglandin E_2 (PGE_2), which are potent activators of osteoclasts, is enhanced by co-stimulation with a NOD1 or NOD2 ligand and a TLR2 or TLR4 ligand in HDPCs (Marriott *et al.*, 2005). Moreover, the production of osteoclastogenic cytokines, including IL-1β, IL-6, and IL-8, is accelerated by co-stimulation with NOD1 or NOD2 and TLR2 or TLR4 ligands *via* increased expression of TNF receptor-associated factor 6 (TRAF6) in periodontal

ligament cells (Tang *et al.*, 2011). In addition, MDP synergistically enhances LPS-mediated osteoclast differentiation through increased RANKL expression in osteoblasts (Yang *et al.*, 2005). These findings suggest that MDP might play an important role in pulpal immune responses, destruction of pulpal architecture, and, ultimately, periapical bone destruction. However, the mechanisms involved in dental pulp destruction under pro-inflammatory stimuli are not fully understood. Here we show that NOD2 functions as a key mediator of MDP signals, which inhibits odontoblast differentiation and promotes osteoclastogenic cytokine expression. Our results indicate a novel role for NOD2 in dental pulp cell regulation that might be applied to the treatment of destructive pulp disease.

MATERIALS & METHODS

Chemicals and Reagents

α-modified Eagle medium (α-MEM), fetal bovine serum (FBS), and penicillin/streptomycin were purchased from Gibco BRL Co. (Grand Island, NY, USA). L-ascorbic acid, β-glycerophosphate, and muramyl dipeptide (MDP) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Lipofectamine 2000 was obtained from Invitrogen Life Technology (Grand Island, NY, USA). The small interfering RNAs (siRNA) against NOD2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Primary antibody against NOD2 and Triptolide (PG490) were purchased from Santa Cruz Biotechnology.

Cell Culture

Immortalized HDPCs, established by stable transfection of human telomerase catalytic component (hTERT), were kindly provided by Prof. Takashi Takata (Hiroshima University, Japan). A detailed characterization of this cell line concerning odontoblast differentiation was thoroughly described previously (Kitagawa *et al.*, 2007). For odontoblast differentiation, HDPCs were cultured in osteogenic medium (OM, 50 μg/mL ascorbic acid and 10 mM β-glycerophosphate, and 0.1 μM dexamethasone) for 14 days as previously described (Wei *et al.*, 2007).

Alkaline Phosphatase Assay

ALP activity was measured with *p*-nitrophenyl phosphate (3 mM final concentration) as the substrate in 0.7 M 2-amino-methyl-1-propanol, pH 10.3, and 6.7 mM MγCl₂. Absorbance was measured at 410 nm by means of an enzyme-linked immunosorbent assay reader (Beckman Coulter, Fullerton, CA, USA).

Alizarin Red Staining

After 14 days of culture, HDPCs were fixed in 70% ice-cold ethanol for 1 hr and rinsed with distilled water. Cells were then stained with 40 mmol/L alizarin red S (pH = 4.2) for 10 min under conditions of gentle agitation. The images of alizarin red S staining were photographed with a digital camera, and the intensity of staining was quantified with the ImageJ program

(ver. 1.40; available on the National Institutes of Health [USA] Website).

NOD2 siRNA Transfection

Small interfering RNAs (siRNAs) were used for transient gene knockdown studies. The sequences of the sense and antisense strands of the human NOD2 siRNA were as follows: 5′-GAUGAAGUUGACCUCCUCA-3′ (sense) and 5′-UGAGG AGGUCAACUUCAUC-3′ (antisense). The control-siRNA (Catalog No. SN-1003) was purchased from Bioneer (Bioneer Corporation, Daejeon, South Korea). HDPCs were transfected with siRNAs duplexes (100 nmol/mL) for 24 hr with Lipofectamine 2000 (Gibco; Invitrogen Ltd, Paisley, UK) according to the manufacturer's instructions.

RNA Isolation and Reverse-transcriptase-Polymerase Chain-reaction (RT-PCR)

The total RNA of cells was extracted with Trizol reagent (Life Technologies, Gaithersburg, MD, USA) according to the manufacturer's instructions. A 1-μg quantity of isolated RNAs from each culture was reverse-transcribed with oligo (dT)₁₅ primers with AccuPower RT PreMix (Bioneer). Next, the generated cDNAs were amplified with AccuPower PCR PreMix (Bioneer). Primer sequences are listed in the Appendix Table.

Western Blot Analysis

Cells were solubilized in ice-cold 1% Triton X-100 lysis buffer. Proteins (30 μg) were mixed with an equal volume of 2 × sodium dodecyl sulphate (SDS) sample buffer, boiled for 5 min, and then resolved by SDS-polyacrylamide gel electrophoresis (12% acrylamide) and transferred to nitrocellulose membranes. The protein bands were visualized by the enhanced chemiluminescence (ECL) system according to the manufacturer's instructions and exposed to x-ray film.

Preparation of Conditioned Medium

HDPCs (2×10^6) were seeded on 100-mm culture dishes and cultured for 14 days in OM to allow for odontoblast differentiation. To obtain conditioned medium (CM) from these cells, we incubated the cultured HDPCs with fresh α-MEM (without MDP) for the last 24 hr of culture. To normalize the differences between cell densities due to proliferation during the culture period, we collected cells from each plate and determined total DNA content/plate (spectrophotometric absorbance, 260 nm). The CM was then normalized for DNA content between samples by the addition of α-MEM medium.

Osteoclast Differentiation Assays

Mouse bone-marrow-derived macrophages (BMMs) obtained from five-week-old female ICR mice (outbred CD-1® mice; Charles River Laboratories, Seoul, South Korea) were used as osteoclast precursor cells for *in vitro* osteoclastogenesis experiments. Briefly, mouse whole bone marrow cells, isolated by
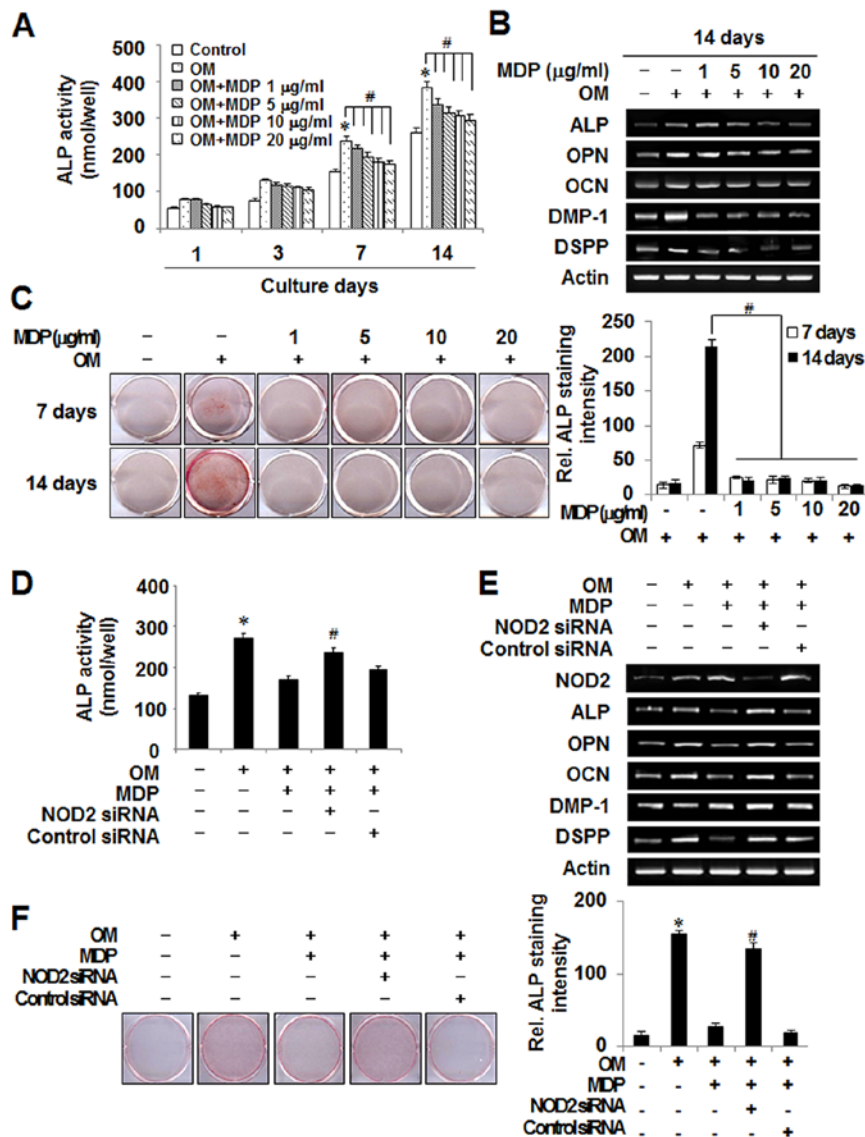
Figure 1. MDP treatment negatively regulates odontoblast differentiation through NOD2 in HDPCs. **(A)** HDPCs were cultured with MDP at concentrations ranging from 0 to 20 µg/mL for the indicated days in osteogenic media (10 mM β-glycerophosphate, 10^{-7} M dexamethasone, and 50 µg/mL L-ascorbic acid). The unstimulated (Control) and OM-alone-treated cells (OM) were used as negative and positive controls, respectively. ALP activity was determined. **(B)** The mRNA expression of osteoblast/odontoblast differentiation markers was evaluated by RT-PCR. **(C)** The mineralized nodule formation was analyzed by alizarin red staining. The intensity of alizarin red staining was quantified as described in Materials & Methods. **(D)** Control siRNA- or NOD2 siRNA-transfected cells were incubated for 24 hr and further cultured with MDP (10 µg/mL) for 7 days in OM. At the end of culture, ALP activity was assessed. **(E)** Control siRNA- or NOD2 siRNA-transfected HDPCs were cultured in the presence of MDP (10 µg/mL) for 7 days in osteogenic media (OM). At the end of culture, the mRNA expression of osteoblast/odontoblast markers was examined by RT-PCR. **(F)** Mineralized nodule formation was analyzed by alizarin red staining of the cells treated as in (E). The bar graph indicates intensity of staining. *Statistically significant difference *vs.* control, $p < .05$. #Statistically significant difference in OM alone, $p < .05$. Similar data were obtained from 3 independent experiments. This figure is available in color online at http://jdr.sagepub.com.

cultured for 3 days in the presence of M-CSF (30 ng/mL) on Petri dishes. After 3 days' culture, BMMs became adherent and were used as osteoclast precursor cells. Upon two-day treatment of BMMs with 30 ng/mL M-CSF and 100 ng/mL RANKL, > 80% of the cells became mononuclear TRAP-positive cells (pre-osteoclasts). To evaluate the osteoclastogenic activity of CM from HDPCs, we treated the CM mixture (60% CM plus 40% fresh α-MEM without M-CSF & RANKL) with pre-osteoclast-stage cells and further cultured it for up to 4 days to achieve mature osteoclast differentiation. After 4 days of culture, cells were stained for TRAP activity with a leukocyte acid phosphatase kit (Sigma). Cells with 3 or more nuclei were counted as multinucleated mature osteoclasts.

Statistical Analysis

The data are expressed as mean ± SD of at least 3 independent experiments. Statistical significance was evaluated by one-way analysis of variance with the SPSS (Version 11.0; SPSS, Chicago, IL, USA) computer program.

RESULTS

MDP, an NOD2 Agonist, Negatively Regulates the Odontoblastic Differentiation of HDPCs

To determine whether the NOD2 ligand MDP affects the odontoblast differentiation of HDPCs, we assessed ALP activity, mRNA expression of odontoblast differentiation markers, and mineralized nodule formation after MDP treatment. As shown in Figs. 1A and 1B, MDP reduced ALP activity and odontoblast (*e.g.*, DMP-1 and DSPP)/osteoblast (*e.g.*, ALP, OPN, and OCN) gene mRNA expression in a dose-dependent manner. The maximal down-regulation of ALP activity and odontoblast/osteoblast marker gene expression was achieved at 10 or 20 µg/mL of MDP; however, the mineralized nodule formation was almost completely inhibited from 1 µg/mL MDP treatment (Fig. 1C). Interestingly, the MDP treatment profoundly increased expression of NOD2, TLR2, and TLR4, which are known to be expressed as pattern-recognition receptors in HDPCs (Appendix

flushing the marrow space of tibiae, were incubated overnight on culture dishes in α-MEM supplemented with 10% FBS. After attached cells were discarded, floating cells were further

Fig.). Next, we tested whether the inhibitory effect of MDP was mediated by NOD2 by introducing NOD2-targeting siRNA into HDPCs. The ALP activity, odontoblast/osteoblast mRNA expression, and mineralized nodule formation recovered in the NOD2-siRNA transfected HDPCs in the presence of MDP (Figs. 1D-1F). Analysis of these data indicates that NOD2 mediates the inhibitory effect of MDP on odontoblast differentiation.

MDP Reduced Phosphorylation of MAPKs through the Induction of MKP-1

For odontoblast differentiation, the activation of mitogen-activated protein kinases (MAPKs) is essential (Jaiswal *et al.*, 2000; Su *et al.*, 2010). NOD2 is known to induce MAPK signaling (Inohara and Nuñez, 2003). To further delineate the mechanism for the suppression of odontoblast differentiation by MDP, we investigated the phosphorylation of MAPKs with MDP treatment. Notably, the MDP treatment prominently decreased phosphorylation of ERK, p38, and JNK (Fig. 2A). Since these 3 major MAPKs are negatively regulated by a common dual-specificity phosphatase known as MAPK phosphatase 1 (MKP-1) (Owens and Keyse, 2007; Huang and Tan, 2012), we hypothesized that MKP-1 expression might be up-regulated by MDP. As expected, MKP-1 protein expression increased in the MDP-treated cultures (Fig. 2A), and, consistent with this result, treatment with an MKP-1 inhibitor, Triptolide, restored MAPK phosphorylation in the presence of MDP (Fig. 2B). In addition, the reduced ALP activity in the MDP-treated HDPCs was rescued by MKP-1 inhibition (Fig. 2C). Therefore, we suggest that the MDP suppression of odontoblast differentiation was mediated by MKP-1 and the subsequent dephosphorylation of MAPKs.

MDP Augments Osteoclastogenic Cytokine Expression in HDPCs

Since MDP is one of the peptidoglycan constituents of Gram-positive/-negative bacteria (Girardin *et al.*, 2003), and since bacterial infection-triggered pulpitis goes with osteoclastic bone resorption (Adachi *et al.*, 2007; Silva *et al.*, 2012), we investigated the effect of MDP on osteoclastogenic cytokine expression in HDPCs. MDP treatment enhanced the expression of osteoclastogenic cytokines, which are known to support osteoclast differentiation (Fig. 3A). Importantly, RANKL and M-CSF,
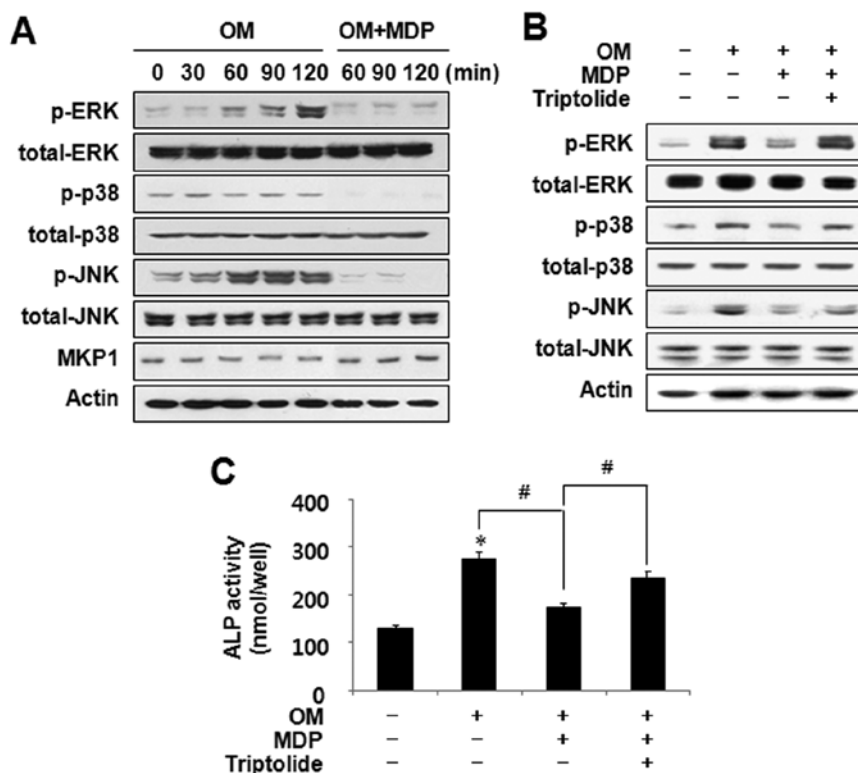

Figure 2. MDP treatment reduced phosphorylation of MAPKs *via* the induction of MAPK phosphatase (MKP)-1 in HDPCs. **(A)** HDPCs were stimulated with MDP at 10 µg/mL for 120 min in osteogenic media (OM) with 10 mM β-glycerophosphate, 10^{-7} M dexamethasone, and 50 µg/mL L-ascorbic acid. The phosphorylation of MAPKs and the expression of MKP-1 were determined by Western blotting. **(B)** HDPCs were pre-treated with MKP-1 inhibitor (Triptolide, TP 0.5 mM) for 4 hr, and stimulated with MDP for 120 min. The phosphorylation of MAPKs was examined by Western blotting. **(C)** HDPCs were cultured in the presence of MDP or Triptolide for 14 days. At the end of culture, ALP activity was examined. *Statistically significant difference *vs.* control, $p < .05$. #Statistically significant difference in OM alone, $p < .05$. Similar data were obtained from 3 independent experiments.

the two key factors essential for osteoclast formation, were also increased in the presence of MDP (Fig. 3B). On the contrary, the mRNA expression of a soluble decoy receptor for RANKL, known as osteoprotegerin (OPG), was substantially reduced upon MDP treatment. Thus, it seems that the presence of MDP provided favorable conditions for osteoclastogenesis through HDPCs. To verify the enhanced osteoclastogenic ability of MDP-treated HDPCs, pre-osteoclasts were allowed to differentiate into mature osteoclasts with the conditioned medium (CM), prepared from MDP-treated HDPCs. According to the RT-PCR results, MDP-treated CM increased osteoclast formation in an MDP dose-dependent manner (Figs. 3C, 3D). Analysis of these data suggests that MDP could enhance osteoclastogenesis by altering the state of supporting HDPCs.

NOD2 Silencing Diminishes the MDP-mediated Osteoclastogenic Cytokine Expression in HDPCs

Since the expression of NOD2 was significantly increased upon MDP treatment and the inhibitory effect of MDP on odontoblast differentiation was exerted by NOD2, the role of NOD2 for the
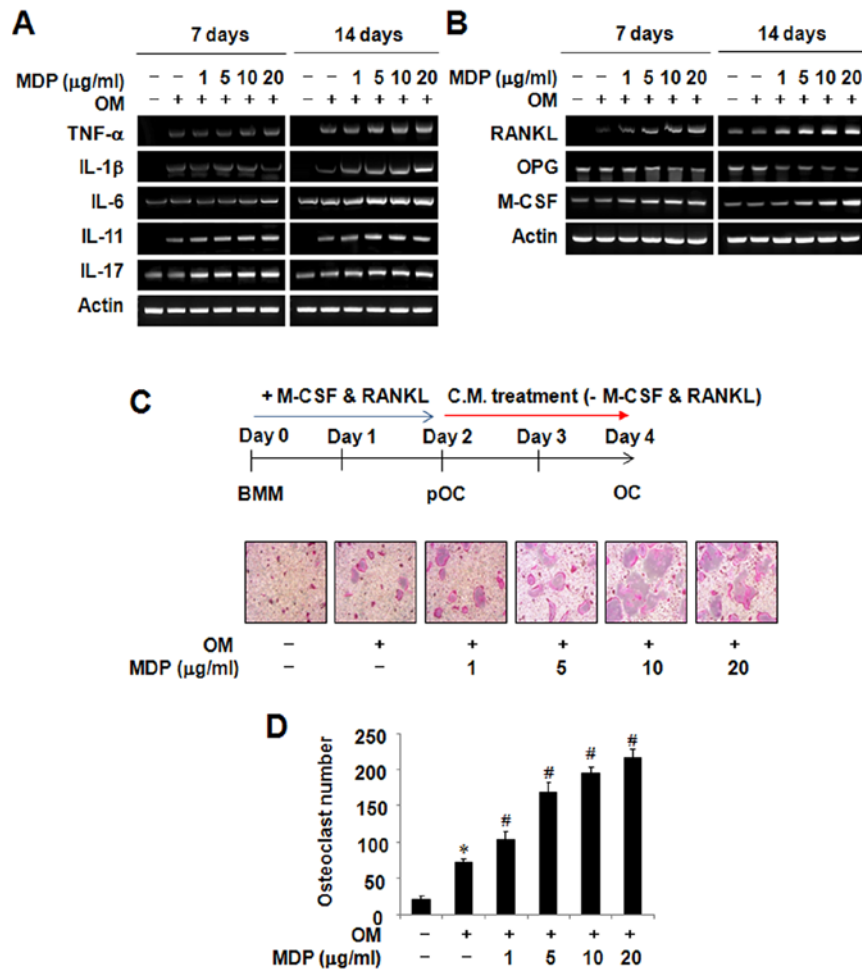
Figure 3. The treatment of MDP potentiates osteoclastogenic cytokine expression in human dental pulp cells. **(A, B)** The effects of MDP on the mRNA expression of osteoclastogenic cytokine in HDPCs were examined by RT-PCR analysis as described in Materials & Methods. **(C)** The osteoclastogenic effects of conditioned medium (CM) from MDP-treated HDPCs were evaluated by an *in vitro* osteoclast differentiation system. Human dental pulp cells were cultured in the presence of indicated concentrations of MDP for 14 days, and CM was prepared as described in Materials & Methods. At the end of culture, cells were stained for TRAP activity. **(D)** The numbers of osteoclasts/well were counted. Similar data were obtained from 3 independent experiments. *Statistically significant difference *vs.* control, $p < .05$.

DISCUSSION

The pathogenesis of pulpal and periapical lesions is largely attributed to host inflammatory responses caused by the bacterial infection of the pulp cavity and root canals (Kakehashi *et al.*, 1965). Periapical bone loss reflects enhanced osteoclast activity and suppressed osteoblast activity during the host response to bacteria. Therefore, we hypothesized that MDP, a peptidoglycan constituent of both Gram-positive/-negative bacteria (Girardin *et al.*, 2003), might be involved in negative odontoblast differentiation as well as up-regulation of osteoclastogenic cytokine expression in HDPCs; the culmination of this process is periapical alveolar bone destruction or internal pulp resorption.

HDPCs are the major constituent of pulp tissue and are capable of playing a pivotal role in wound-healing processes, pathological alterations, and the production of various inflammatory cytokines. In addition, HDPCs are considered odontoblast progenitor cells that are capable of proliferating and differentiating into odontoblast-like cells that conduct dentin formation (Fitzgerald *et al.*, 1990). Our results show that treatment with the NOD2 ligand MDP decreased ALP activity, mineralized nodule formation, and the expression of odontoblast markers in HDPCs. These results are similar to those of previous reports in mouse osteoblasts (Pelt *et al.*, 2002) and rat bone marrow cells (Andreou *et al.*, 2004) treated with LPS, a bacterial component.

Recently, it has been reported that HDPCs express stimulatory (RANKL, M-CSF) and inhibitory (OPG) factors (Sundqvist *et al.*, 1995), which means that the key molecules in osteoclast progenitor cell expansion and differentiation are both expressed in pulp tissue. Evidence demonstrates that RANKL is expressed in dental pulp tissue in periapical lesions (Zhang and Peng, 2005) and during root resorption (Shapiro *et al.*, 2002). Since RANKL expression could be up-regulated by bone-resorbing factors such as IL-1, IL-6, IL-11, IL-17, PGE$_2$, and parathyroid hormone in osteoblasts (Blair and Athanasou, 2004), we examined the expression of RANKL in HDPCs upon MDP treatment. Interestingly, MDP increased pro-inflammatory cytokine expression (*i.e.*, TNF-α, IL-1β, IL-6, IL-11, and IL-17) as well as RANKL expression in HDPCs (Fig. 3). Although the question of whether MDP up-regulated RANKL expression directly or indirectly through the induction of pro-inflammatory mediators could not be clearly answered by these results, a noticeable increase of osteoclast formation was observed upon treatment

osteoclastogenic cytokine expression in HDPCs was tested by the introduction of NOD2 siRNA into HDPCs. Introduction of siRNA specific for NOD2 reduced osteoclastogenesis-supporting cytokine expression compared with control siRNA-transfected HDPCs (Fig. 4A). More importantly, increased expression of RANKL and M-CSF in the presence of MDP was markedly down-regulated upon knockdown of NOD2 (Fig. 4B), suggesting that NOD2 functions downstream of MDP. To examine the function of NOD2 in osteoclastogenic cytokine expression, we evaluated osteoclastogenic activity of CM from NOD2-silenced HDPCs under an osteoclast differentiation system. Although the addition of MDP resulted in increased osteoclast formation, this effect was abolished by knockdown of NOD2 (Figs. 4C, 4D). Therefore, we conclude that MDP caused elevated osteoclastogenic cytokine expression in HSPCs by mediating NOD2.

with CM prepared from MDP-treated HDPCs (Fig. 3). MDP alone could not induce osteoclast formation in the osteoclast differentiation culture (data not shown), but enhanced osteoclast formation was evident with CM from MDP-treated HDPCs. More importantly, the MDP-mediated osteoclastogenic cytokine expression in HDPCs was reduced by NOD2 knockdown (Fig. 4). To our knowledge, this study is the first demonstration that the MDP-NOD2 pathway is directly involved in osteoclastogenic cytokine expression in HDPCs.

Inohara *et al.* (2005) reported that NOD1 is ubiquitously expressed in numerous cell types, whereas NOD2 is mainly expressed in immune cells. NOD2 regulates the coordination of signaling pathways downstream of bacterial infection by inducing activation of MAPKs, which usually initiates the production of pro-inflammatory cytokines (Inohara and Nuñez, 2003). In this study, we demonstrated that treatment with MDP induced dephosphorylation of p38, ERK, and JNK (Fig. 2) by inducing MKP-1 expression. Since the activation of MAPKs is essential for odontoblast differentiation (Jaiswal *et al.*, 2000; Su *et al.*, 2010), it is likely that the observed reduction of odontoblast differentiation induced by MDP was mainly caused by the direct inhibition of MAPKs. It should be noted that the suppression of odontoblast differentiation by MDP vanished in NOD2-silenced HDPCs (Fig. 1). Thus, analysis of these data adds another line of evidence that the MDP contained in bacteria negatively regulates odontoblast differentiation *via* NOD2.

To summarize, we propose dual roles for NOD2 during odontoblast differentiation and osteoclastogenic cytokine expression in HDPCs. The increased NOD2 expression in MDP-challenged HDPCs results in decreased odontoblast differentiation by increasing MKP-1 protein levels and the subsequent down-regulation of MAPKs. NOD2 also mediates osteoclastogenic cytokine expression in HDPCs in the presence of MDP. Therefore, during the invasion of caries-related bacteria into dentin tissue, MDP results in disastrous bone loss, reflecting enhanced osteoclastogenesis and suppressed odontoblastogenesis. The mechanism described in this study might improve our understanding about the pathogenesis of pulpal and periapical lesions.
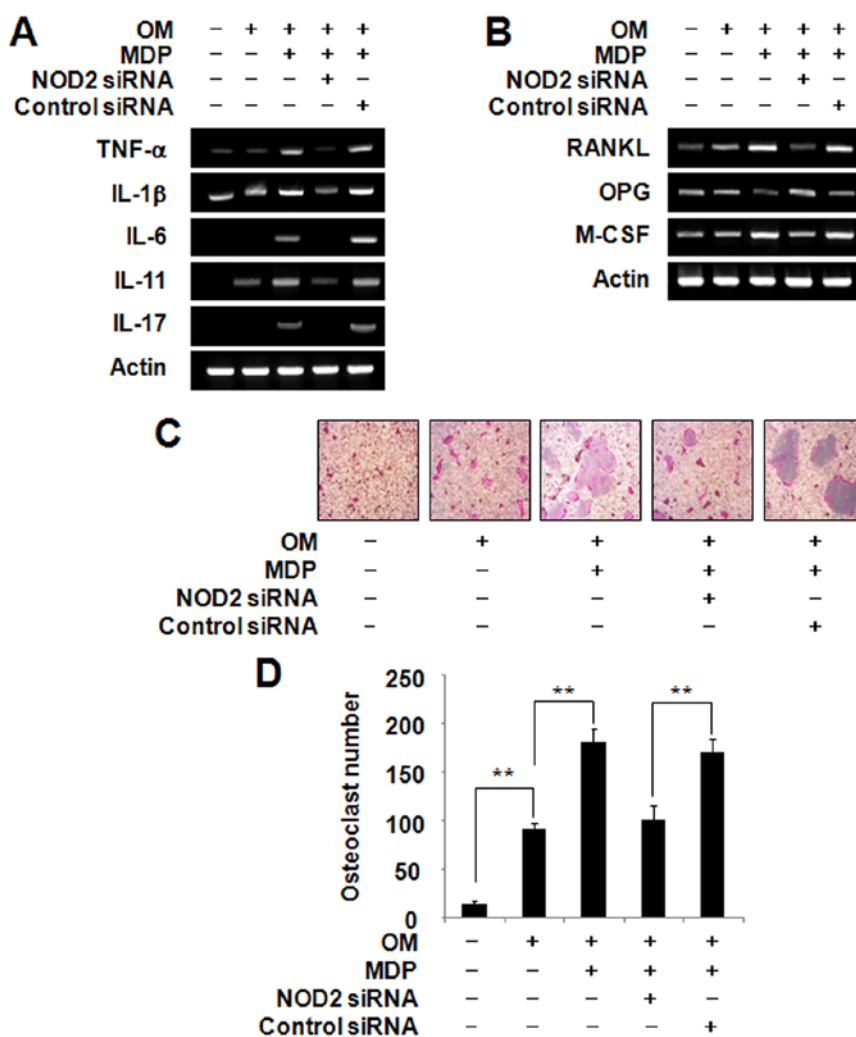

Figure 4. The knockdown of NOD2 reversed MDP-mediated osteoclastogenic cytokine expression in HDPCs. **(A, B)** Control siRNA- or NOD2 siRNA-transfected HDPCs were cultured in the presence of MDP (10 μg/mL) for 7 days in osteogenic media (OM). The mRNA levels of osteoclastogenic cytokines were evaluated by RT-PCR analysis. **(C)** The osteoclastogenic activity of conditioned medium (CM) from NOD2-silenced HDPCs was assessed by a mouse osteoclast differentiation system. HDPCs were transfected with either control siRNA or NOD2 siRNA and subsequently treated with MDP (10 μg/mL) for 14 days. The CM was collected as described in Materials & Methods and treated on pre-osteoclast cells. After 2 days, the cultures were stained for TRAP activity. **(D)** The numbers of osteoclasts/well were counted. *Statistically significant difference *vs.* control, $p < .05$. #Statistically significant difference in OM alone, $p < .05$. Similar data were obtained from 3 independent experiments.

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