ARTICLE
Journal of Cellular Biochemistry 111:1310–1320 (2010)

Journal of Cellular Biochemistry

SIRT1 Modulates High-Mobility Group Box 1-Induced Osteoclastogenic Cytokines in Human Periodontal Ligament Cells

Young-Suk Kim,[1] Young-Man Lee,[1] Jae-Sang Park,[1] Suk-Keun Lee,[2] and Eun-Cheol Kim[1*]

[1]*Department of Oral & Maxillofacial Pathology, School of Dentistry, Wonkwang University, Iksan, Republic of Korea*

[2]*Department of Oral Pathology, College of Dentistry, Kangneung-Wonju National University, Kangneung, Republic of Korea*

ABSTRACT

Bone resorptive cytokines contribute to bone loss in periodontal disease. However, the involvement of SIRT1 in high-mobility group box 1 (HMGB1)-induced osteoclastic cytokine production remains unknown. The aim of this study was to investigate the role of SIRT1 in the responses of human periodontal ligament cells to HMGB1 and to identify the underlying mechanisms. The effect of HMGB1 on osteoclastic cytokine expression and secretion, and the regulatory mechanisms involved were studied by ELISA, reverse transcription-polymerase chain reaction, and Western blot analysis. HMGB1 upregulated the mRNA expression levels of the osteoclastic cytokines tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IL-11, and IL-17. In addition, HMGB1 upregulated RANKL mRNA expression, and SIRT1 mRNA and protein expression. The upregulation of these cytokines by HMGB1 was attenuated by pretreatment with inhibitors of p38 mitogen-activated protein kinase and NF-κB, as well as neutralizing antibodies against Toll-like receptors 2 and 4. Inhibition of SIRT1 by sirtinol or SIRT1 siRNA blocked the HMGB1-stimulated expression of RANKL and cytokines. These results suggest that the inhibition of SIRT1 may attenuate HMGB1-mediated periodontal bone resorption through the modulation of osteoclastogenic cytokine levels in human periodontal ligament cells. J. Cell. Biochem. 111: 1310–1320, 2010.

KEY WORDS: SIRT1; HMGB1; CYTOKINES; RANKL; PERIODONTAL LIGAMENT CELLS

Periodontal disease infection with oral biofilm microorganisms initiates host immune response and signs of periodontitis, including bone resorption [Haffajee et al., 2009]. In inflammatory diseases such as arthritis and in infectious diseases characterized by bone resorption, it is thought that receptor activator of nuclear factor (NF)-kappa B ligand (RANKL) plays a key role in inducing osteoclasts and bone resorption [Verdrengh et al., 2010]. Periodontal disease is an inflammatory disorder of the periodontium and is characterized by the destruction of periodontal tissues, including periodontal ligament (PDL), cementum, alveolar bone, and gingiva [Page and Schroeder, 1976]. The PDL is a specialized, vascular, and highly cellular connective tissue. PDL cells consist of heterogeneous cell populations with fibroblasts, cementoblasts, osteoblasts, osteoclasts, and endothelial cells [Lekic et al., 2001]. Previous studies indicate that PDL cells possess the potential to form mineralized nodules in vitro, to express bone-related markers, such as bone sialoprotein (BSP) and osteocalcin (OCN), and to express characteristic bone regulatory hormone responses [Carnes et al., 1997; Lekic et al., 2001]. Thus, it is very likely that PDL cells include cell populations at different stages of differentiation and lineage commitment. In addition, human periodontal ligament cells (hPDLCs) express and secrete osteoprotegerin (OPG) and RANKL, suggesting that they play a role in alveolar bone metabolism [Hasegawa et al., 2002; Crotti et al., 2003]. Our previous studies demonstrated that RANKL can be induced in hPDLCs by various stimuli, including H_2O_2, nicotine, and substance P [Pi et al., 2007; Lee et al., 2009a, 2010].

In addition to RANKL, several pro-inflammatory cytokines, often referred to as osteoclastogenic cytokines, have been implicated in osteoclast differentiation. Among these, tumor necrosis factor (TNF)-α and interleukin (IL)-1 contribute to osteoclast differentiation/activation associated with inflammatory osteolysis [Kobayashi et al., 2000]. Other cytokines, including IL-6, IL-11, and IL-17, have been reported to support osteoclast formation by upregulating the

Grant sponsor: National Research Foundation of Korea (NRF); Grant number: 2009-0078526.

*Correspondence to: Prof. Eun-Cheol Kim, DDS, PhD, Department of Oral & Maxillofacial Pathology, Dental College, Wonkwang University, Shinyoungdong 344-2, Iksan City, Jeonbuk 570-749, South Korea.
E-mail: eckwkop@wonkwang.ac.kr

Received 22 April 2010; Accepted 17 August 2010 • DOI 10.1002/jcb.22858 • © 2010 Wiley-Liss, Inc.
Published online 27 August 2010 in Wiley Online Library (wileyonlinelibrary.com).

expression of RANKL, which promotes osteoclast differentiation [Hofbauer et al., 2000]. These cytokines are thought to play an important role in the pathogenesis of periodontitis, as they cause inflammation and destruction of periodontal tissue and resorption of alveolar bone by various biological mechanisms.

High-mobility group box 1 (HMGB1), also known as amphoterin and HMG1, is an abundant 30-kDa non-histone nuclear protein expressed in all eukaryotic cells [Biianchi et al., 1989; Andersson et al., 2000]. HMGB1 acts as an intracellular regulator of transcription and plays a crucial role in the maintenance of DNA functions [Lu et al., 1996]. Extracellular high-mobility-group box 1 released from various cells or necrotic cells [Wang et al., 1999; Scaffidi et al., 2002] and stimulated by LPS or TNF-α acts as a pro-inflammatory cytokine through the multiligand receptor for advanced glycation end-products and toll-like receptors 2 and 4 [Dumitriu et al., 2005; Lotze and Tracey, 2005]. Extracellular high-mobility-group box 1 has been found to play critical roles in the progression of chronic inflammatory diseases, such as septic shock, rheumatoid arthritis, endotoxemia, cancer, and atherosclerotic lesions [Wang et al., 1999; Taniguchi et al., 2003]. The mechanisms through which HMGB1 acts are not yet fully understood; however, it is well established that HMGB1 binds to the receptor for advanced glycation end-products (RAGE), a transmembrane receptor belonging to the immunoglobulin superfamily that is expressed on a wide variety of cells, such as smooth muscle cells, neurons, endothelial cells, monocytes, osteoblasts, human gingival fibroblasts (HGF), and epithelial cells [Erlandsson et al., 2004; Katz et al., 2005; Charoonpatrapong et al., 2006]. It was recently reported that gingival crevicular fluid from patients with periodontitis contained HMGB1, whereas fluid from healthy patients did not, suggesting a potential role of HMGB1 protein in disease progression [Morimoto et al., 2008]. In addition, HGF can be a source of HMGB1, by both active secretion and passive release, and HMGB1 from HGF may contribute to periodontal tissue destruction [Feghali et al., 2009]. Furthermore, HMGB1 released from RANKL-stimulated mouse bone marrow macrophages is necessary for RANKL-induced differentiation of osteoclast precursors in vivo and in vitro [Yang et al., 2007a].

SIRT1 is a class III histone/protein deacetylase and a member of the silent information regulator (Sir2) family. It has been implicated in the regulation of lifespan, gene expression, cellular metabolism, and cellular stress responses [Haigis and Guarente, 2006]. Recently, SIRT1 was shown to exert anti-inflammatory effects by deacetylating modified lysine residues in transcriptional regulators, notably NF-κB, a master transcription factor involved in the regulation of pro-inflammatory cytokines [Salminen et al., 2008]. The anti-inflammatory action of SIRT1 is further supported by a previous study in which resveratrol, an activator of SIRT1, inhibited cigarette smoke-induced IL-8 release in a monocyte–macrophage cell line [Yang et al., 2007b], and cytokine-mediated cytotoxicity in RIN cells [Lee et al., 2009b]. By contrast, bronchial inflammation and levels of IL-4, IL-5, and IL-13 in the lungs of ovalbumin-sensitized mice were significantly reduced by the SIRT1 inhibitor sirtinol [Kim et al., 2010]. Although these studies have not yielded a consensus, it seems likely that SIRT1 plays an important role in inflammatory processes. However, the expression of SIRT1 in HMGB1-treated hPDLCs and its role in the regulation of osteoclastogenic cytokines remain unknown. The aim of the present study was to investigate the role of SIRT1 in HMGB1-stimulated generation of osteoclastic cytokines in hPDLCs and to elucidate the signaling mechanisms involved.

MATERIALS AND METHODS

REAGENTS

Resveratrol, sirtinol, and recombinant HMGB1 (rHMGB1) protein were purchased from Sigma–Aldrich Chemical Co (St. Louis, MO). Human TNF-α and IL-1β ELISA Kit were purchased from R&D Systems, Inc. (Minneapolis, MN). Mouse anti-human TLR2 and TLR4 antibodies were from eBioscience (San Diego, CA). Affinity purified polyclonal antibodies against mouse TLR2, TLR4, IκB-α, NF-κB, p65, and β-actin monoclonal antibodies were obtained from Santa Cruz Biotechnology (Delaware Avenue, CA). Anti-human TLR2 and TLR4 monoclonal antibodies (Abcam, Cambridge, UK) were used for in vitro neutralization studies in hPDL cells. Antibodies (Abs) against phospho-ERK (p-ERK), ERK, phospho-p38 (p-p38), p38, phospho-JNK (p-JNK), and JNK were purchased from Cell Signaling, Inc. (Beverly, MA).

CELL CULTURE

We used the immortalized hPDL cell lines by transfection with telomerase catalytic subunit *hTERT* gene [Kitagawa et al., 2006]. Cells were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2 at 37°C. For experiments, the cells were seeded onto culture dishes, and then cultured in DMEM containing 10% FBS for 3 days until 70% confluent, and media were then replaced by serum-free medium in order to minimize any serum-induced effects on PDL cells. Subsequently, the cells were exposed to HMGB1 for 24 h. Additional experiments were conducted by simultaneous addition of HMGB1 and polymyxin B (10 μg/ml; Sigma–Aldrich Chemical Co.) for 48 h. All treatments were performed in triplicate.

MEASUREMENT BY ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

The concentrations of TNF-α and IL-1β in the culture supernatants were determined by an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Rochester, MN) according to the manufacturer's recommended procedure. The plates were read at 450 nm on a microplate reader (Molecular Devices, Sunnyvale, CA).

RNA ISOLATION AND REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION (RT-PCR)

Total RNA was prepared from cells by using the Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Reverse transcription of 1 μg/ml of total RNA to cDNA was performed by using the AccuPower RT PreMix kit (Bioneer Corporation, Daejeon, South Korea). The primers used for cDNA amplification and PCR conditions were as follows: for TNF-α, 5′-CCTCTGG CCCAGGCAGTCAGA-3′ (sense) and 5′-GGCGTTTG-GGAAGGTTGGAT-3′ (antisense); for IL-1β, 5′-GGATATGGAGCAA-CAAGTGG-3′ (sense) and 5′-ATGTACCAGTTG GGGAACTG-3′

(antisense); for IL-6, 5′-ATGAACTCCTTCTCCACAAGC-3′ (sense) and 5′-CTACATTTGCCGAAGAGCCC-3′ (antisense); for IL-11, 5′-CTGCTGCTGCTGAAGACTCGGCTGTGA-3′ (sense) and 5′-ATGGGGAAGAGCCAGGGCAGAAGTCTGT-3′ (antisense); for IL-17, 5′-TCCCCAGTTGATTGGAAGA-3′ (sense) and 5′-AGTCAAACCTTCCTTCTTGGA-3′ (antisense); for SIRT1, 5′-GCAACATCTTATGATTGGCACA-3′ (sense) and 5′-AAATACCATCCCTTGACCTGAA-3′ (antisense); for RANKL, 5′-GCCAGTGGGAGATGTTAG-3′ (sense) and 5′-TTAGCTGCAAGTTTTCCC-3′ (antisense); for OPG, 5′-TGCAGTACGTCAAGCAGGAG-3′ (sense) and 5′-TGACCTCTGTGAAAACAGC-3′ (antisense); for M-CSF, 5′-ATGACAGACAGGTGGAACTGCCAGTGTAGAGG-3′ (sense) and 5′-TCACACAACTTCAGTAGGTTCAGGTGA TGGGC-3′ (antisense); for β-actin, 5′-CATGGATGATGATATCGCCGC-3′ (sense) and 5′-ACATGATCTGGGTCATCTTCT-3′ (antisense). The reaction conditions for PCR were 30–35 cycles of denaturation at 95°C for 30 s, annealing at 55–60°C for 30 s, and extension at 72°C for 1 min. The PCR products were resolved on 1.5% agarose gel and stained with ethidium bromide.

REAL-TIME POLYMERASE CHAIN REACTION ANALYSIS

Real-time PCR was performed on cDNA samples using the SYBR Green system (Bio-Rad, Richmond, CA). Primers used were SIRT1, sense 5′-TCAGTGTCATGGTTCCTTTGC-3′ and antisense 5′. The following general real-time PCR protocol was employed: denaturation for 10 min at 95°C, 40–50 cycles of a four segmented amplification and quantification program, a melting step by slow heating from 60 to 99°C with a rate of 0.1°C/s and continuous fluorescence measurement, and a final cooling down to 40°C. Crossing point (CP) values were acquired by using the second derivative maximum method of the LightCycler software 3.3 (Roche, Burlington, NC). Real-time PCR efficiencies were acquired by amplification of a standardized dilution series, and slopes were determined using LightCycler Software 3.3 (Roche).

MEASUREMENTS OF ENDOTOXIN ACTIVITY

The endotoxin activities of rHMGB1 preparations were determined using the Limulus amoebocycle lysate assay kit (BioWhittaker, Walkersville, MD) according to the manufacturer's recommendation. Briefly, 50 μl of the standard or samples was dispensed into the appropriate microplate wells. Then, 50 μl of the Limulus amoebocyte lysate solution was added and the microplate was incubated for 10 min at 37 ± 1°C. One hundred microliters of the substrate solution were pipetted and the reaction was stopped after 6 min by adding 100 μl of 25% acetic acid. The absorbance was read at 405 nm in a microplate reader and a standard curve plotted. The results were expressed in EU/ml.

SIRT1 SIRNA TRANSFECTION

siRNA-annealed oligonucleotide duplexes for SIRT1 (sequence 5′ → 3′ sense: GAUGAAGUUGACCUCCUCAtt; antisense: UGAGGAGGUCAACUUCAUCtt) and negative control (catalog no. SN-1003) were purchased from Bioneer Corporation, and hPDLCs were transfected using Lipofectamine 2000 (Gibco; Invitrogen Ltd, Paisley, UK) following the manufacturer's instructions.

WESTERN BLOTTING

Cells (1×10^6) from each set of experiments were harvested and washed twice in cold Tris-buffered saline. Cells were solubilized in ice-cold 1% Triton X-100 lysis buffer. After 30 min on ice, the lysates were clarified by centrifugation. Proteins (20 μg) were resolved by SDS–PAGE (10% acrylamide), transferred to nitrocellulose membranes, and probed with specific Abs (diluted 1/1,000), followed by incubation with secondary HRP-conjugated Ab (1/5,000). Proteins were detected by enhanced chemiluminescence system according to the manufacturer's instructions and exposed to X-ray.

STATISTICAL ANALYSIS

The data are expressed as the means ± SD. The statistical analyses of the data were performed by one-way ANOVAs followed by a multiple-comparison Turkey's tests with the use of the SPSS program (SPSS 12.0; SPSS GmbH, Munich, Germany). Statistical significance was determined at $P < 0.05$.

RESULTS

EFFECTS OF HMGB1 ON THE EXPRESSION OF OSTEOCLASTOGENIC CYTOKINES AND OSTEOCLASTOGENIC REGULATORY MOLECULES IN HPDLCS

To investigate whether HMGB1 could induce osteoclastogenic cytokines in hPDLCs, cells were stimulated with various doses of HMGB1, and TNF-α and IL-1β expression was measured by ELISA and RT-PCR (Fig. 1A,B). After 24 h of HMGB1 stimulation, hPDLCs secreted significant amounts of TNF-α and IL-1β; a HMGB1 concentration of 1 μg/ml gave the largest increase (Fig. 1A). TNF-α and IL-1β mRNA and protein expression were upregulated by HMGB1 in a time-dependent fashion, with cytokine accumulation peaking at 24 h post-treatment (Fig. 1B,C). The effects of HMGB1 on the expression of osteoclast differentiation-associated cytokines in hPDLCs were analyzed by RT-PCR. The upregulation of IL-6, IL-11, and IL-17 mRNA levels was detected within 2 h of treatment with 1 μg/ml HBGB1, and the levels had peaked by 24 h (Fig. 1C).

We also used RT-PCR to examine the effects of HMGB1 on the mRNA expression levels of the osteoclastogenic regulatory molecules RANKL, OPG, and macrophage colony-stimulating factor (M-CSF) in cultured hPDLCs (Fig. 1D). Following treatment with 1 μg/ml HMGB1, RANKL mRNA expression increased in a time-dependent manner, between 4 and 48 h. By contrast, the expression of the anti-osteoclastic genes OPG and M-CSF did not change, regardless of the duration of HMGB1 treatment.

EFFECTS OF SIRT1 ACTIVATION OR INHIBITION ON HMGB1-INDUCED SIRT1 MRNA AND PROTEIN EXPRESSION IN HPDLCS

To understand the role of SIRT1 in HMGB1-induced cell signaling processes, we first assessed the expression of SIRT1 mRNA by real-time PCR (Fig. 2A). As shown in Figure 2A, SIRT1 mRNA levels increased time-dependently in hPDLCs after treatment with 1 μg/ml HMGB1. The induction of SIRT1 mRNA was evident as early as 12 h post-treatment.

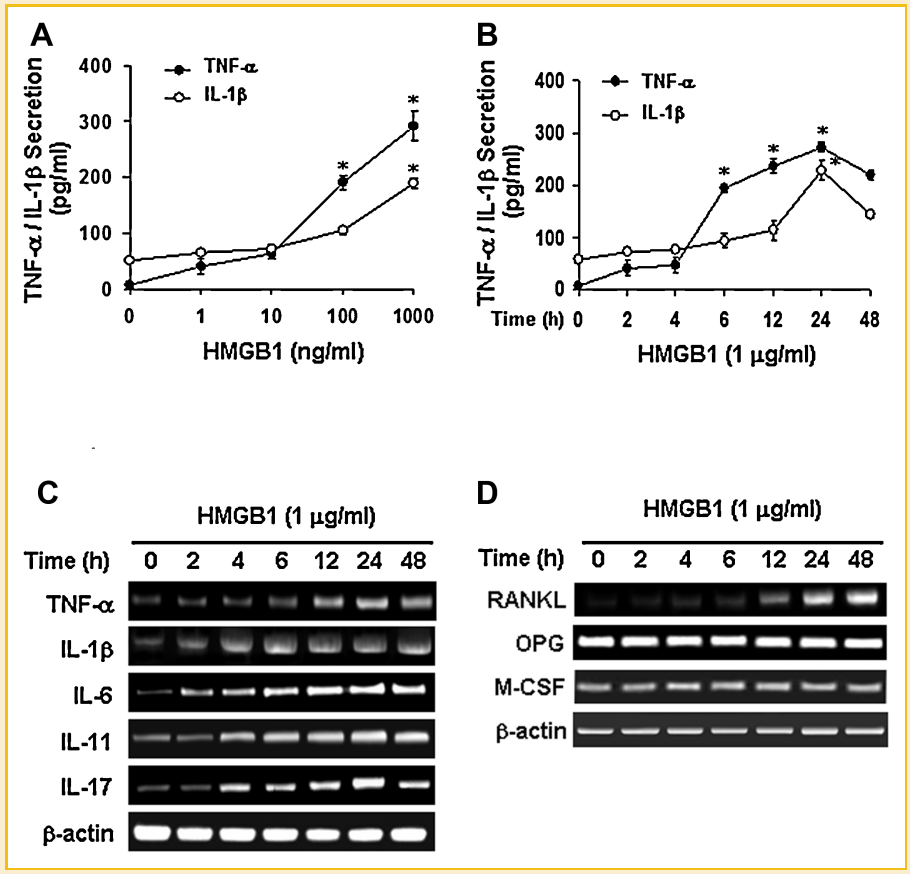

Fig. 1. Effects of HMGB1 on secretion (A,B) and mRNA expression (C,D) of osteoclastogenic cytokines in hPDLCs. A: Cells were stimulated with different concentrations of HMGB1 for 24 h. B: Cells were incubated with 1 μg/ml HMGB1 for the indicated periods of time (0–48 h). TNF-α and IL-1β levels in cellular supernatants were quantified by ELISA (A,B). Data are means ± SD of three independent experiments performed in triplicate. *Significantly different from control, $P < 0.05$. C,D: Representative results of RT-PCR analyses of cytokine and osteoclastogenic regulatory molecule mRNA expression. Similar results were obtained in three independent experiments.

Next, hPDLCs were pretreated with either resveratrol (an SIRT1 activator) or sirtinol (an SIRT1 inhibitor), and HMGB1-induced SIRT1 mRNA and protein levels were analyzed (Fig. 2A,B). Pretreatment with 50 μM resveratrol for 1 h significantly enhanced HMGB1-induced SIRT1 mRNA and protein levels, whereas 25 μM sirtinol attenuated HMGB1-induced SIRT1 mRNA and protein expression.

EFFECTS OF ENDOTOXIN ON HMGB1-INDUCED OSTEOCLASTOGENIC CYTOKINES AND SIRT1 MRNA EXPRESSION IN HPDLCS

To distinguish possibility the observed effect of rHMGB1 was due to some contaminants present in the rHMBG1 preparation, we determined the functional characteristics as well as endotoxin contents. The endotoxin contents of the HMGB1 preparations (1 μg) were determined and always found to be below 0.12 EU/ml (Fig. 3A). In addition, the effects of the endotoxin inhibitor polymysin B were evaluated on osteoclastogenic cytokines (Fig. 3B). Addition of 10 μg/ml polymyxin B had no effects on HMGB1-stimulated RANKL, TNF-α, IL-1β, IL-6, IL-11, IL-17, and SIRT1 expression.

EFFECTS OF SIRT1 ACTIVATION OR INHIBITION ON HMGB1-INDUCED EXPRESSION OF OSTEOCLASTOGENIC CYTOKINES AND RANKL IN HPDLCS

To identify the role of SIRT1 in interacting the osteoclastogenic cytokines producing effects of HMGB1 in hPDL cells, we initially investigated the effects of SIRT1 activation and inhibition. Pretreatment of hPDLCs with sirtinol inhibited HMGB1-induced TNF-α and IL-1β mRNA expression and secretion in a time-dependent manner (Fig. 4A,B). Pretreatment with resveratrol enhanced the effects of HMGB1. Furthermore, pretreatment with sirtinol or resveratrol, respectively, abolished or increased HMGB1-mediated induction of IL-6, IL-11, and IL-17 mRNA expression (Fig. 4C). Similarly, HMGB1-enhanced expression of RANKL mRNA was suppressed by sirtinol pretreatment but increased by resveratrol (Fig. 4D).

EFFECT OF SIRT1 SIRNA ON HMGB1-INDUCED CYTOKINE AND RANKL MRNA EXPRESSION

To further confirm the role of SIRT1 in HMGB1's induction of cytokine and osteoclastogenic factor expression, we knocked down SIRT1 with a specific siRNA. As shown in Figure 5B, SIRT1 siRNA

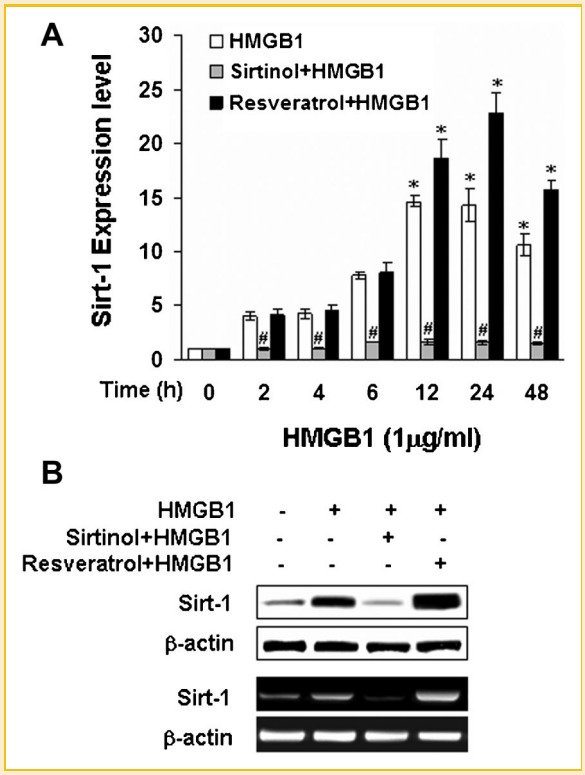

Fig. 2. Effects of sirtinol and resveratrol on HMGB1-induced SIRT1 mRNA and protein expression in hPDLCs. Cells were pretreated with 25 μM sirtinol (SIRT1 inhibitor) or 50 μM resveratrol (SIRT1 activator) for 1 h and then stimulated with HMGB1 for the indicated periods of time (0–48 h). A: Expression of SIRT1 mRNA was evaluated by real-time PCR. Data are means ± SD of three independent experiments performed in triplicate. *Significantly different from control, $P < 0.05$. #Significantly different from HMGB1-treated group, $P < 0.01$. B: SIRT1 protein and mRNA levels were evaluated by Western blot analysis and RT-PCR, respectively. Similar results were obtained in three independent experiments.

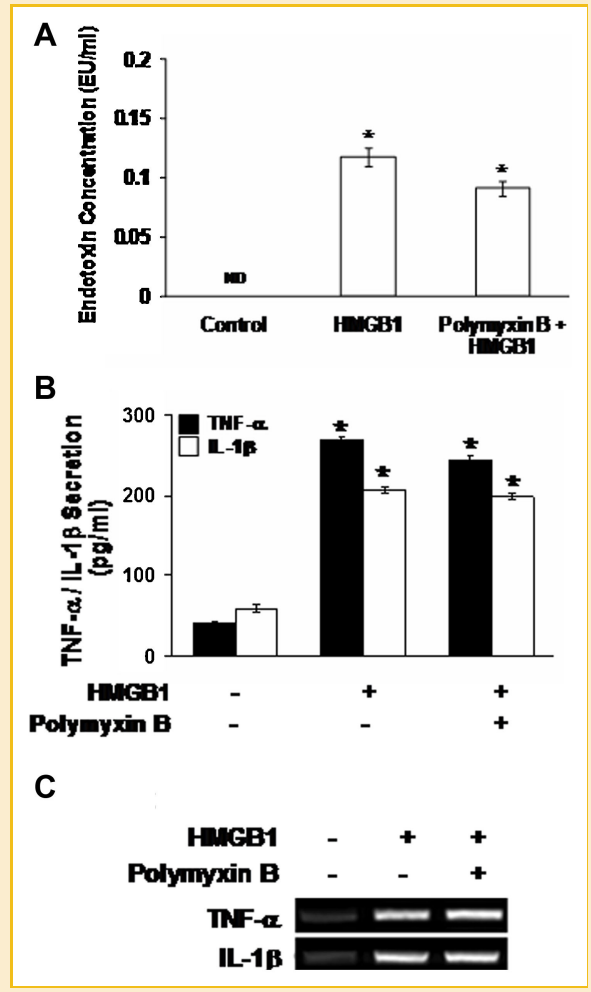

Fig. 3. Endotoxin assay in recombinant HBGB1 (A) and effect of polymyxin B on rHBGB1 preparation-mediated osteoclastogenic cytokines production (B) and mRNA expression (C). Recombinant HMGB1 was tested for the presence of LPS by using the Limulus lysate endotoxin assay kit. Cells were incubated with 1 μg/ml HMGB1 in the presence of 10 μg/ml polymyxin B for 48 h. Similar results for mRNA were obtained in three independent experiments. Control is DMEM media. ND, no detected.

successfully knocked down SIRT1 expression in hPDLCs, and SIRT1 knockdown blocked the HMGB1-induced expression of the mRNAs encoding RANKL (Fig. 5C) and the osteoclastogenic cytokines TNF-α, IL-1β, IL-6, IL-11, and IL-17 (Fig. 5A,B).

EFFECTS OF HMGB1 ON EXPRESSION OF TLR2 AND TLR4 PROTEINS

Recently, HMGB1 was shown to signal via TLR2 and/or TLR4 [Park et al., 2004]. To elucidate the role of SIRT1 in HMGB1 signaling, we initially examined whether HMGB1 influences the expression of TLR2 and TLR4 in hPDLCs. Following exposure to HMGB1, TLR2 and TRL4 protein levels were increased (Fig. 6A). The TLR2 and TLR4 levels peaked 2 and 4 h, respectively, after HMGB1 treatment. Furthermore, HMGB1-induced TLR2 and TLR4 protein expression was inhibited by sirtinol (Fig. 6B) and enhanced by resveratrol (Fig. 6C) in a time-dependent manner.

EFFECT OF BLOCKING TLR2 AND TLR4 ON HMGB1-INDUCED EXPRESSION OF CYTOKINES AND RANKL

To examine the role of TLRs in HMGB1 signaling, hPDLCs were preincubated for 30 min with neutralizing monoclonal antibodies against TLR2 and TLR4 and then treated with HMGB1 (1 μg/ml). Antibody specificity was assessed by incubating with control IgG from non-immunized animals. The anti-TLR4 and anti-TLR2 antibodies (10 μg/ml) blocked the induction by HMGB1 of osteoclastogenic cytokine secretion (Fig. 7A) and mRNA expression (Fig. 7B), and of RANKL mRNA expression (Fig. 7C), suggesting that TLR2 and TLR4 are required for HMGB1 to elicit these responses in hPDLCs.

INVOLVEMENT OF NF-*K*B AND MAP KINASE IN HMGB1-INDUCED EXPRESSION OF CYTOKINES AND RANKL

To elucidate the molecular basis of the responses to HMGB1, we examined the effects of HMGB1 on the MAP kinase (MAPK) and NF-κB signaling pathways in hPDLCs. Following cell activation with 1 μg/ml HMGB1, the degradation of IκBα protein and the nuclear translocation of p65 (NF-κB) increased in a time-dependent manner

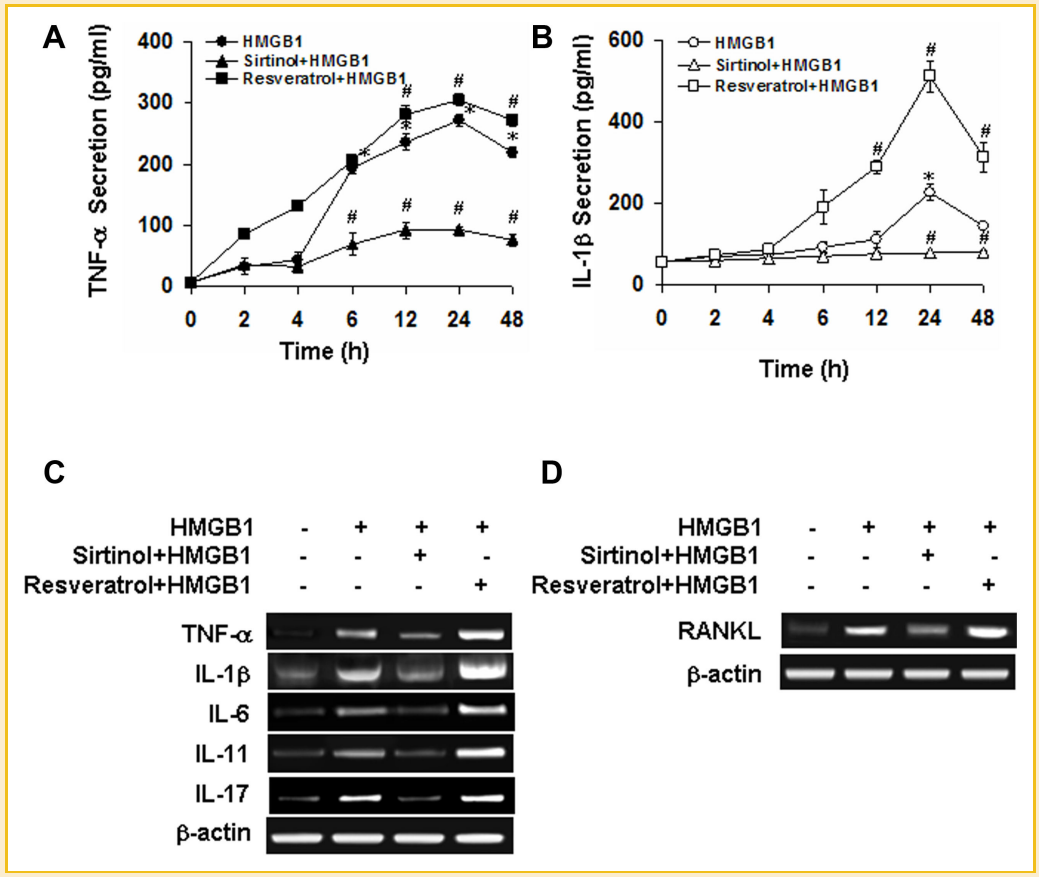

Fig. 4. Effects of SIRT1 activation and inhibition on HMGB1-induced cytokine secretion and expression in hPDLCs. Cells were pretreated with 25 μM sirtinol or 50 μM resveratrol for 1 h and then stimulated with HMGB1 for the indicated periods of time (0–48 h). A,B: TNF-α and IL-1β levels in cellular supernatants were quantified by ELISA. Data are means ± SD of three independent experiments performed in triplicate. *Significantly different from control, $P < 0.05$. #Significantly different from HMGB1-treated group, $P < 0.05$. C,D: Representative results of RT-PCR analyses of cytokine and RANKL mRNA expression. Similar results were obtained in three independent experiments.

(Fig. 8A). As shown in Figure 7B, HMGB1 treatment induced the phosphorylation of p38 MAPK but not ERK or JNK. Given that HMGB1 activated NF-κB and phosphorylated p38 MAPK, we examined the effects of pharmacological inhibitors on HMGB1-induced cytokine expression and secretion. Pretreatment of cells with SB203580 (a p38 MAPK inhibitor) or PDTC (a NF-κB inhibitor) blocked HMGB1-induced secretion of TNF-α and IL-1β (Fig. 8C) and expression of the mRNAs encoding TNF-α, IL-1β, IL-6, IL-11, IL-17 (Fig. 8D), and RANKL (Fig. 8E).

DISCUSSION

Periodontal disease is characterized by chronic inflammation of periodontal tissues and involves the interaction between bacterial invasion and host response resulting in pocket formation, irreversible bone resorption, and subsequently tooth loss. A recent study showed that gingival crevicular fluid from periodontal patients contained HMGB1, whereas that from healthy patients did not, suggesting a potential role of the HMGB1 protein in sustaining inflammation and hence contributing to disease progression [Morimoto et al., 2008]. In addition, HMGB1 is expressed at high levels in macrophages and differentiating osteoclasts and is sufficient to induce osteoclastogenesis in preosteoclasts [Yang et al., 2007a]. However, the effects of HMGB1 on the release of osteoclastogenic cytokines and the signaling pathways involved remain largely unknown. Because SIRT1 is associated with anti-inflammatory and anti-immune responses [Yang et al., 2007b; Salminen et al., 2008], we postulated that SIRT may be involved in the responses of hPDLCs to HMGB1.

The PDL is a source of pluripotential cells and molecular factors controlling cellular events in the surrounding tissues [Bellows et al., 1999]. Generally, PDL cells are a heterogeneous population, consisting mainly of fibroblasts and osteoblasts. In the present study, we used immortalized hPDL cell line from Kitagawa et al. [2006], which showed low alkaline phosphatase (ALP) and mineralization activity, and did not express bone-like matrix proteins such as OCN and BSP. However, Kitagawa et al. [2006] demonstrated that F-spondin increased the expressions of ALP, OCN, and BSP mRNA, and ALP activity in immortalized hPDL cell line. Recent studies have demonstrated that the upregulation of RANKL was noted in the progression of bone loss in periodontal disease [Liu et al., 2003]. Both OPG and RANKL expression have been identified in PDL cells, with increased RANKL expression in

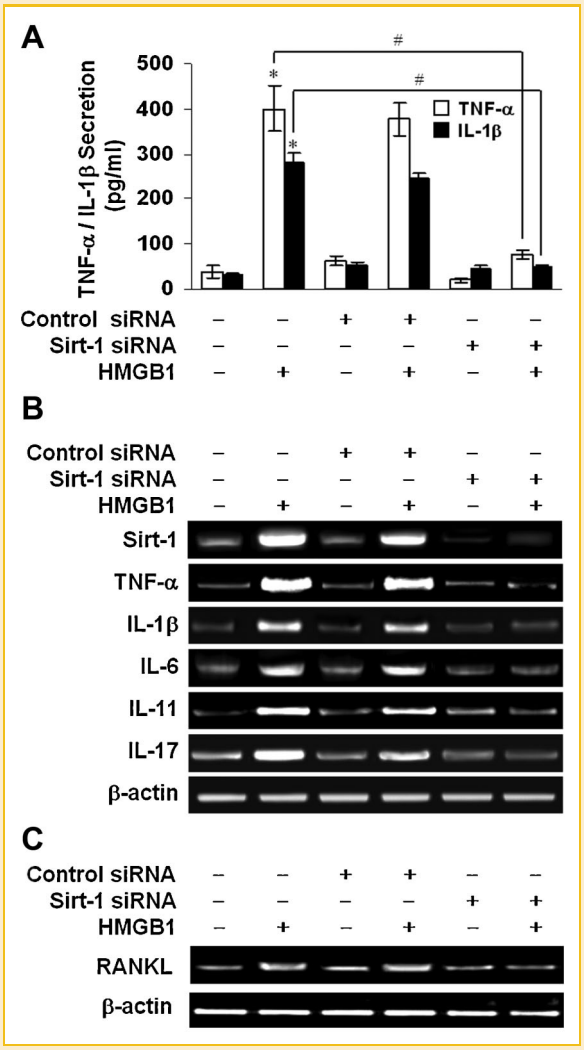

Fig. 5. Effects of SIRT1 siRNA on HMGB1-induced cytokine and RANKL expression in hPDLCs. Cells were transiently transfected with empty vector or SIRT1 siRNA and then stimulated with HMGB1 for 24 h. A: TNF-α and IL-1β levels in cellular supernatants were quantified by ELISA. Data are means ± SD of three independent experiments performed in triplicate. *Significantly different from control, $P < 0.05$. Representative results of RT-PCR analyses of cytokine (B) and RANKL (C) mRNA expression. Similar results for mRNA were obtained in three independent experiments.

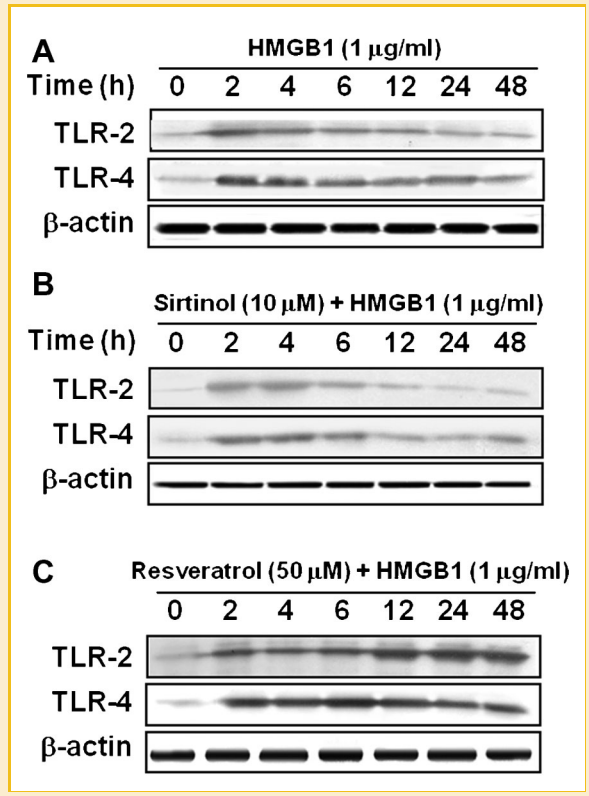

Fig. 6. (A) Effect of HMGB1 on the expression of TLR2 and TLR4 proteins in hPDLCs. Effect of 10 mM sirtinol (B) and 50 mM resveratrol (C) on HMGB1-induced expression of TLR2 and TLR4 proteins.

PDL cells associated with inflammatory mediators, lipopolysaccharides (LPS), prostaglandin E2 (PGE2), and IL-1, as well as increased bone resorptive activity [Hasegawa et al., 2002; Crotti et al., 2003; Wada et al., 2004]. It is therefore also important to observe the expression of RANKL and other osteoclastogenic cytokines in PDL cells that could be related to the biological function of osteoclasts. Thus, we focused on the response of hPDLCs to HMGB1 since the alveolar bony response is primarily mediated by the PDL. This study demonstrates the cellular and molecular mechanisms against HMGB1-induced osteoclastic cytokine expression in hPDLCs, focusing on the roles of SIRT1.

Focal osteolysis, a major complication of periodontal disease, is characterized by accelerated bone resorption resulting from local secretion of pro-inflammatory cytokines. The present study we focused on the cytokines TNF-α, IL-1, IL-6, IL-11, and IL-17, which are collectively referred to as osteoclastogenic cytokines, because they promote RANKL expression in bone marrow stromal cells and osteoblasts [Hofbauer et al., 2000; Kobayashi et al., 2000]. HMGB1 in turn induces the production of pro-inflammatory mediators such as TNF-α and IL-1β [Andersson et al., 2000]. In addition, monocytes and macrophages exposed to TNF-α and IL-1β have been shown to release HMGB1 [Wang et al., 1999; Andersson et al., 2000]. In this study, we showed that treating hPDLCs with HMGB1 increased the expression of TNF-α, IL-1β, IL-6, IL-11, and IL-17 mRNA. These results are in accordance with previous reports that HMGB1 activated the synthesis of TNF-α, IL-1α, IL-1β, IL-1Rα, IL-6, IL-8, MIP-1α, and MIP-1β, but not IL-10 or IL-12, in monocytes [Andersson et al., 2000].

Although RANKL has been shown to stimulate the release of HMGB1 from macrophages and osteoclasts [Hofbauer et al., 2000], the secretion of osteoclastogenic regulatory molecules from HMGB1-treated cells has not previously been reported. To confirm our finding that HMGB1 enhanced the release of osteoclastogenic cytokines, we showed that HMGB1 upregulated the expression of RANKL mRNA but had no significant effect on the OPG and M-CSF mRNA levels. Our observations are in line with previous reports that pro-inflammatory cytokines such as TNF-α and IL-6 are released from HMGB1-stimualted osteoblasts and osteoclasts [Hofbauer et al., 2000; Yang et al., 2007a].

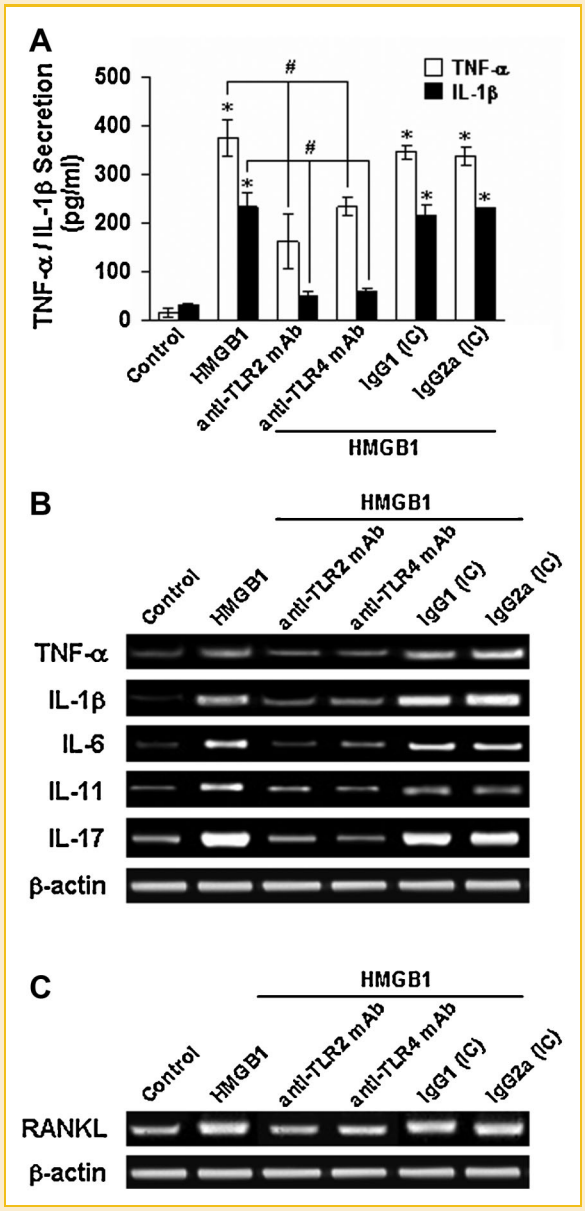

Fig. 7. Effect of blocking TLR2 and TLR4 on HMGB1-induced expression of cytokines and RANKL mRNA in hPDLCs. Cells were pretreated with anti-TLR2 (10 μg/ml) or anti-TLR4 monoclonal antibody (10 μg/ml), or isotype-matched control mouse IgG antibody (10 μg/ml) for 1 h and then stimulated with HMGB1 for 24 h. A: TNF-α and IL-1β levels in cellular supernatants were quantified by ELISA. Data are means ± SD of three independent experiments. B,C: Representative results of RT-PCR analyses of cytokine and RANKL mRNA expression. RT-PCR data are from three independent experiments.

It has been reported that antibiotic polymyxin B can bind to lipid A portion of LPS and interfere with LPS function [Morrison and Jacobs, 1976]. In our preliminary experiment, polymyxin B in concentrations ranging from 1 to 30 μg/ml was added to cells 30 min before the addition of *Escherichia coli* LPS (10–100 ng/ml). The media were collected 4 h later and analyzed for endotoxin. In the present study, 10 μg/ml of polymyxin B was chosen because we found that this dose gives maximal inhibition on LPS-induced endotoxin and cytokines (data not shown). Moreover, the dose (10 μg/ml) of polymyxin B used in this study has been shown to effectively suppressed the LPS (100 ng/ml) induced endotoxin in macrophage [Lo et al., 2009] and LPS (33–0.3 ng/ml) induced TNF-α level in THP1 cells [Zimmermann et al., 2004]. The endotoxin activity of rhHMGB1 by Limulus assay and effect of polymyxin B on HMGB1-induced cytokines (Fig. 3) demonstrated that endotoxin contamination of the recombinant HMGB1 preparation does not appear to be the basis for our observations.

Extracellular stimuli such as cigarette smoke, cytokines, UV radiation, and H_2O_2 have been shown to decrease SIRT1 levels and increase NF-κB-dependent release of pro-inflammatory mediators [Yang et al., 2007B; Cao et al., 2008; Lee et al., 2009b], although the molecular mechanisms by which SIRT1 is activated are yet to be fully elucidated. It seems unlikely that a single, ubiquitous mechanism underlies SIRT1 activation or repression, as its regulation differs among tissues and with different stimuli [Chen et al., 2008]. Both chronic calorie restriction and acute fasting have been shown to increase the SIRT1 protein level and activity [Cohen et al., 2004]. In addition, SIRT1 is activated in response to oxidative stress, DNA damage, nitric oxide, cGMP, adiponectin, pyruvate, H_2O_2, and lonomycin [Nisoli et al., 2005; Rodgers et al., 2005; Kim et al., 2007]. The present study demonstrated that HMGB1 increases SIRT1 mRNA and protein expression in hPDLCs, as has been shown in various other cell types [Nisoli et al., 2005; Rodgers et al., 2005; Kim et al., 2007]. Furthermore, preincubation of hPDLCs with sirtinol or resveratrol blocked or enhanced, respectively, the induction of SIRT1 expression by HMGB1. These results indicate that SIRT1 is involved in the response of hPDLCs to HMGB1.

Accumulating evidence implicates SIRT1 in the modulation of anti-inflammatory responses [Yang et al., 2007b; Salminen et al., 2008]. However, the role of SIRT1 in HMGB1-mediated induction of osteoclastogenic cytokines requires clarification. Previous studies have demonstrated that resveratrol-induced SIRT1 activation or adenoviral-mediated SIRT1 overexpression blocked the expression and release of pro-inflammatory cytokines in response to environmental stresses [Yang et al., 2007b; Rajendrasozhan et al., 2008; Lee et al., 2009b]. In the present study, we showed that downregulating SIRT1 expression via SIRT1 siRNA or blocking SIRT1 activity via sirtinol prevented HMGB1-mediated induction of TNF-α, IL-1β, IL-6, IL-11, IL-17, and RANKL mRNA expression. This result is consistent with the previous finding that sirtinol reduced the increased numbers of airway inflammatory cells, bronchial hyperresponsiveness, and the increased local IL-4, IL-5, and IL-13 mRNA expression in a murine allergic airway disease model [Kim et al., 2010]. In the present study, the SIRT1 activator resveratrol enhanced HMGB1-induced expression of inflammatory cytokines and RANKL in hPDLCs. These results suggest that SIRT1 mediates the increased expression of osteoclastogenic cytokines and RANKL in HMGB1-treated hPDLCs and that SIRT1 inhibition may protect against deleterious effects of HMGB1.

TLRs are involved in not only inflammatory responses but also host defense mechanisms. The binding of HMGB1 to RAGE, TLR2, or TLR4 leads to the recruitment of inflammatory cells and the release of pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6, from neutrophils and macrophages [Park et al., 2004]. In support of previous findings that HMGB1 signals through both TLR2 and TLR4

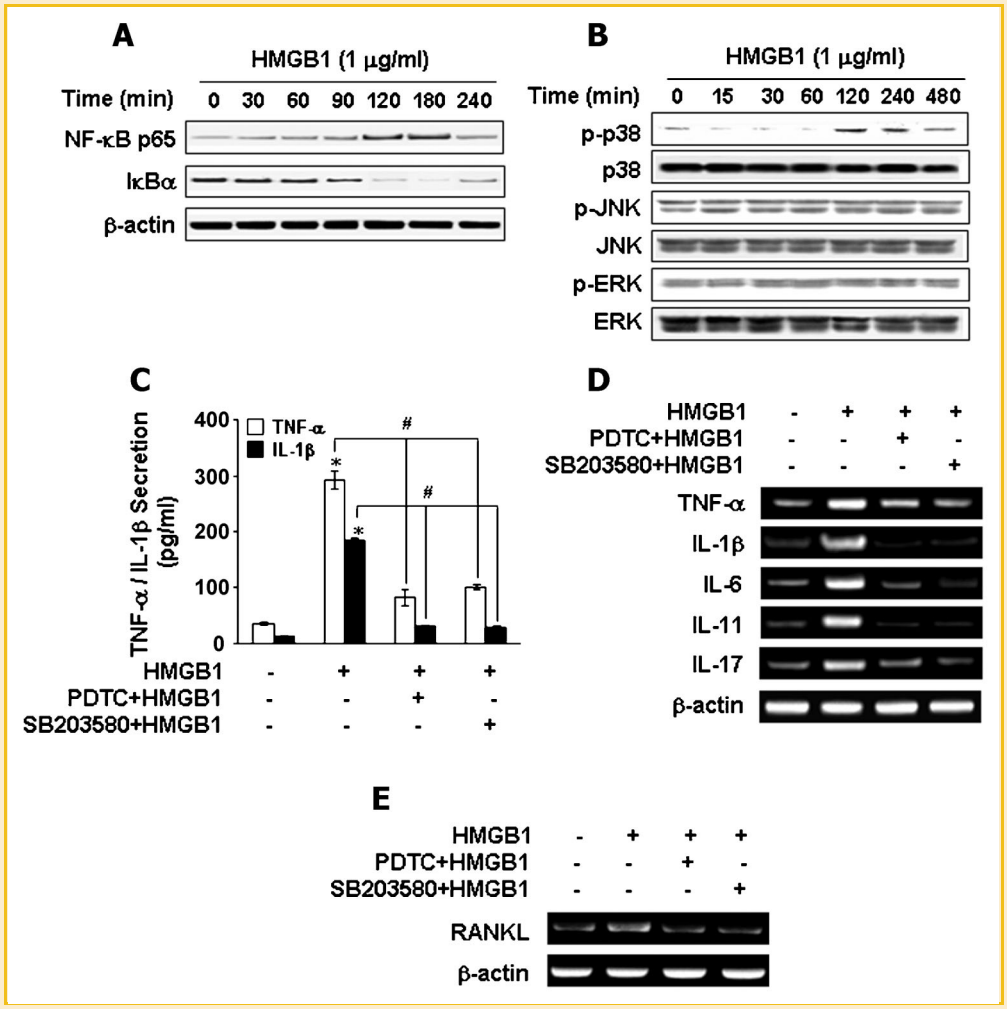

Fig. 8. Contribution of MAPK and NF-κB signaling to HMGB1-induced cytokine expression and secretion in hPDLCs. NF-κB and MAPK activities were determined, respectively, by (A) the degradation of IκBα protein and nuclear translocation of p65 (NF-κB) and (B) the phosphorylation of MAPK, following incubation of hPDLCs with 1 μg/ml HMGB1 for the indicated periods of time. C–E: Effects of the p38 MAPK and NF-κB inhibitor on cytokine mRNA expression and secretion and RANKL mRNA expression in HMGB1-treated hPDLCs. C: TNF-α and IL-1β levels in cellular supernatants were quantified by ELISA. *Significantly different from control, $P < 0.05$. #Significantly different from HMGB1-treated group, $P < 0.05$. D,E: Representative results of RT-PCR analyses of cytokine and RANKL mRNA expression. RT-PCR data are from three independent experiments.

in human neutrophils and macrophages [Park et al., 2004], we found that TLR4 and TLR2 expression was increased in hPDLCs exposed to HMGB1 and that neutralizing anti-TLR2 and anti-TLR4 antibodies specifically inhibited HMGB1-induced expression and secretion of osteoclastogenic cytokines and expression of RANKL.

In cultured macrophages, neutrophils, and Caco-2 epithelial cells, HMGB1 activated several MAP kinases, resulting in the subsequent activation of NF-κB [Sappington et al., 2002; Park et al., 2003]. In this study, we demonstrated that the phosphorylation of p38 MAPK and activation of NF-κB were increased in hPDLCs treated with HMGB1. Furthermore, pretreatment with the NF-κB inhibitor PDTC or the p38 MAPK inhibitor SB203580 effectively suppressed the induction by HMGB1 of osteoclastogenic cytokines. Similar findings have been reported in endothelial cells, in which inhibition of NF-κB and p38 MAPK reduced HMGB1-induced IL-8 release [Fiuza et al., 2003].

To our knowledge, this study is the first to demonstrate that HMGB1 induces osteoclastogenic cytokines in hPDLCs via the activation of p38 MAPK, NF-κB, TLR2, and TLR4. SIRT1 inhibition blocked HMGB1-stimulated cytokine expression in hPDLCs. In view of the key role attributed to osteoclastogenic cytokines in the progression and severity of periodontitis, SIRT1 inhibition may represent a novel mechanism for modulating the inflammatory responses associated with periodontal disease.

ACKNOWLEDGMENTS

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MEST) (No. 2009-0078526).

REFERENCES

Andersson U, Wang H, Palmblad K, Aveberger AC, Bloom O, Erlandsson-Harris H, Janson A, Kokkola R, Zhang M, Yang H, Tracey KJ. 2000. High mobility group 1 protein (HMG-1) stimulates proinflammatory cytokine synthesis in human monocytes. J Exp Med 192:565–570.

Bellows CG, Reimers SM, Heersche JN. 1999. Expression of mRNAs for type-I collagen, bone sialoprotein, osteocalcin, and osteopontin at different stages of osteoblastic differentiation and their regulation by 1,25 dihydroxyvitmain D3. Cell Tissue Res 297:249–259.

Biianchi ME, Beltrame M, Paonessa G. 1989. Specific recognition of cruciform DNA by nuclear protein HMG1. Science 243:1056–1059.

Cao C, Lu S, Kivlin R, Wallin B, Card E, Bagdasarian A, Tamakloe T, Wang WJ, Song X, Chu WM, Kouttab N, Xu A, Wan Y. 2008. SIRT1 confers protection against UVB- and H(2)O(2)-induced cell death via modulation of p53 and JNK in cultured skin keratinocytes. J Cell Mol Med 13:3632–3643.

Carnes DL, Maeder CL, Graves DT. 1997. Cells with osteoblastic phenotypes can be explanted from human gingiva and periodontal ligament. J Periodontol 68:701–707.

Charoonpatrapong K, Shah R, Robling AG, Alvarez M, Clapp DW, Chen S, Kopp RP, Pavalko FM, Yu J, Bidwell JP. 2006. HMGB1 expression and release by bone cells. J Cell Physiol 207:480–490.

Chen D, Bruno J, Easlon E, Lin SJ, Cheng HL, Alt FW, Guarente L. 2008. Tissue-specific regulation of SIRT1 by calorie restriction. Genes Dev 22: 1753–1757.

Cohen HY, Miller C, Bitterman KJ, Wall NR, Hekking B, Kessler B, Howitz KT, Gorospe M, de Cabo R, Sinclair DA. 2004. Calorie restriction promotes mammalian cell survival by inducing the SIRT1 deacetylase. Science 305: 390–392.

Crotti T, Smith MD, Hirsch R, Soukoulis S, Weedon H, Capone M, Ahern MJ, Haynes D. 2003. Receptor activator NF kappa B ligand (RANKL) and osteoprotegerin (OPG) protein expression in periodontitis. J Periodontal Res 38:380–387.

Dumitriu IE, Baruah P, Valentinis B, Voll RE, Herrmann M, Nawroth PP, Arnold B, Bianchi ME, Manfredi AA, Rovere-Querini P. 2005. Release of high mobility group box 1 by dendritic cells controls T cell activation via the receptor for advanced glycation end products. J Immunol 174:7506–7515.

Erlandsson Harris H, Andersson U. 2004. Minireview: The nuclear protein HMGB1 as a proinflammatory mediator. Eur J Immunol 34:1503–1512.

Feghali K, Iwasaki K, Tanaka K, Komaki M, Machigashira M, Ishikawa I, Izumi Y. 2009. Human gingival fibroblasts release high-mobility group box-1 protein through active and passive pathways. Oral Microbiol Immunol 24: 292–298.

Fiuza C, Bustin M, Talwar S, Tropea M, Gerstenberger E, Shelhamer JH, Suffredini AF. 2003. Inflammation-promoting activity of HMGB1 on human microvascular endothelial cells. Blood 101:2652–2660.

Haffajee AD, Teles RP, Patel MR, Song X, Veiga N, Socransky SS. 2009. Factors affecting human supragingival biofilm composition. I. Plaque mass. J Periodontal Res 44:511–519.

Haigis MC, Guarente LP. 2006. Mammalian sirtuins—Emerging roles in physiology, aging, and calorie restriction. Genes Dev 20:2913–2921.

Hasegawa T, Yoshimura Y, Kikuiri T, Yawaka Y, Takeyama S, Matsumoto A, Oguchi H, Shirakawa T. 2002. Expression of receptor activator of NF-kappa B ligand and osteoprotegerin in culture of human periodontal ligament cells. J Periodontal Res 37:405–411.

Hofbauer LC, Khosla S, Dunstan CR, Lacey DL, Boyle WJ, Riggs BL. 2000. The roles of osteoprotegerin and osteoprotegerin ligand in the paracrine regulation of bone resorption. J Bone Miner Res 15:2–12.

Katz J, Caudle RM, Bhattacharyya I, Stewart CM, Cohen DM. 2005. Receptor for advanced glycation end product (RAGE) upregulation in human gingival fibroblasts incubated with nornicotine. J Periodontol 76:1171–1174.

Kim D, Nguyen MD, Dobbin MM, Fischer A, Sananbenesi F, Rodgers JT, Delalle I, Baur JA, Sui G, Armour SM, Puigserver P, Sinclair DA, Tsai LH. 2007. SIRT1 deacetylase protects against neurodegeneration in models for Alzheimer's disease and amyotrophic lateral sclerosis. EMBO J 26:3169–3179.

Kim SR, Lee KS, Park SJ, Min KH, Choe YH, Moon H, Yoo WH, Chae HJ, Han MK, Lee YC. 2010. Involvement of sirtuin 1 in airway inflammation and hyperresponsiveness of allergic airway disease. J Allergy Clin Immunol 125:449–460.

Kitagawa M, Kudo Y, Iizuka S, Ogawa I, Abiko Y, Miyauchi M, Takata T. 2006. Effect of F-spondin on cementoblastic differentiation of human periodontal ligament cells. Biochem Biophys Res Commun 349:1050–1056.

Kobayashi K, Takahashi N, Jimi E, Udagawa N, Takami M, Kotake S, Nakagawa N, Kinosaki M, Yamaguchi K, Shima N, Yasuda H, Morinaga T, Higashio K, Martin TJ, Suda T. 2000. Tumor necrosis factor alpha stimulates osteoclast differentiation by a mechanism independent of the ODF/RANKL–RANK interaction. J Exp Med 191:275–286.

Lee HJ, Pi SH, Kim Y, Kim HS, Kim SJ, Kim YS, Lee SK, Kim EC. 2009a. Effects of nicotine on antioxidant defense enzymes and RANKL expression in human periodontal ligament cells. J Periodontol 80:1281–1288.

Lee JH, Song MY, Song EK, Kim EK, Moon WS, Han MK, Park JW, Kwon KB, Park BH. 2009b. Overexpression of SIRT1 protects pancreatic beta-cells against cytokine toxicity by suppressing the nuclear factor-kappaB signaling pathway. Diabetes 58:344–351.

Lee HJ, Jeong GS, Pi SH, Lee SI, Bae WJ, Kim SJ, Lee SK, Kim EC. 2010. Heme oxygenase-1 protects human periodontal ligament cells against substance P-induced RANKL expression. J Periodontal Res 45:367–374.

Lekic P, Rojas J, Birek C, Tenenbaum H, McCulloch CA. 2001. Phenotypic comparison of periodontal ligament cells in vivo and in vitro. J Periodont Res 36:271–279.

Liu D, Xu JK, Figliomeni L, Huang L, Pavlos NJ, Rogers M, Tan A, Price P, Zheng MH. 2003. Expression of RANKL and OPG mRNA in periodontal disease: Possible involvement in bone destruction. Int J Mol Med 11: 17–21.

Lo HM, Chen CL, Tsai YJ, Wu PH, Wu WB. 2009. Thrombin induces cyclooxygenase-2 expression and prostaglandin E2 release via PAR1 activation and ERK1/2- and p38 MAPK-dependent pathway in murine macrophages. J Cell Biochem 108:1143–1152.

Lotze MT, Tracey KJ. 2005. High-mobility group box 1 protein (HMGB1): Nuclear weapon in the immune arsenal. Nat Rev Immunol 5:331–342.

Lu J, Kobayashi R, Brill SJ. 1996. Characterization of a high mobility group 1/2 homolog in yeast. J Biol Chem 271:33678–33685.

Morimoto Y, Kawahara KI, Tancharoen S, Kikuchi K, Matsuyama T, Hashiguchi T, Izumi Y, Maruyama I. 2008. Tumor necrosis factor-alpha stimulates gingival epithelial cells to release high mobility-group box 1. J Periodontal Res 43:76–83.

Morrison DC, Jacobs DM. 1976. Binding of polymyxin B to the lipid A portion of bacterial lipopolysaccharides. Immunochemistry 13:813–818.

Nisoli E, Tonello C, Cardile A, Cozzi V, Bracale R, Tedesco L, Falcone S, Valerio A, Cantoni O, Clementi E, Moncada S, Carruba MO. 2005. Calorie restriction promotes mitochondrial biogenesis by inducing the expression of eNOS. Science 310:314–317.

Page RC, Schroeder HE. 1976. Pathogenesis of inflammatory periodontal disease. A summary of current work. Lab Invest 34:235–249.

Park JS, Arcaroli J, Yum HK, Yang H, Wang H, Yang KY, Choe KH, Strassheim D, Pitts TM, Tracey KJ, Abraham E. 2003. Activation of gene expression in human neutrophils by high mobility group box 1 protein. Am J Physiol Cell Physiol 284:C870–C879.

Park JS, Svetkauskaite D, He Q, Kim J, Strassheim D, Ishizaka A, Abraham E. 2004. Involvement of TLR2 and TLR4 in cellular activation by high mobility group box 1 protein (HMGB1). J Biol Chem 279:7370–7377.

Pi SH, Kim SC, Kim HT, Lee HJ, Lee SK, Kim EC. 2007. Defense mechanism of heme oxygenase-1 against cytotoxic and receptor activator of NF-κB ligand inducing effects of hydrogen peroxide in human periodontal ligament cells. J Periodontal Res 42:331–339.

Rajendrasozhan S, Yang SR, Kinnula VL, Rahman I. 2008. SIRT1, an anti-inflammatory and antiaging protein, is decreased in lungs of patients with chronic obstructive pulmonary disease. Am J Respir Crit Care Med 177:861–870.

Rodgers JT, Lerin C, Haas W, Gygi SP, Spiegelman BM, Puigserver P. 2005. Nutrient control of glucose homeostasis through a complex of PGC-1a and SIRT1. Nature 434:113–118.

Salminen A, Kauppinen A, Suuronen T, Kaarniranta K. 2008. SIRT1 longevity factor suppresses NF-κB-driven immune responses: Regulation of aging via NF-κB acetylation? BioEssays 30:939–942.

Sappington PL, Yang R, Yang H, Tracey KJ, Delude RL, Fink MP. 2002. HMGB1 B box increases the permeability of Caco-2 enterocytic monolayers and impairs intestinal barrier function in mice. Gastroenterology 123:790–802.

Scaffidi P, Misteli T, Bianchi ME. 2002. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. Nature 418:191–195.

Taniguchi N, Kawahara K, Yone K, Hashiguchi T, Yamakuchi M, Goto M, Inoue K, Yamada S, Ijiri K, Matsunaga S, Nakajima T, Komiya S, Maruyama I. 2003. High mobility group box chromosomal protein 1 plays a role in the pathogenesis of rheumatoid arthritis as a novel cytokine. Arthritis Rheum 48:971–981.

Verdrengh M, Bokarewa M, Ohlsson C, Stolina M, Tarkowski A. 2010. RANKL-targeted therapy inhibits bone resorption in experimental *Staphylococcus aureus*-induced arthritis. Bone 46:752–758.

Wada N, Maeda H, Yoshimine Y, Akamine A. 2004. Lipopolysaccharide stimulates expression of osteoprotegerin and receptor activator of NF-kappa B ligand in periodontal ligament fibroblasts through the induction of interleukin-1 beta and tumor necrosis factor-alpha. Bone 35:629–635.

Wang H, Bloom O, Zhang M, Vishnubhakat JM, Ombrellino M, Che J, Frazier A, Yang H, Ivanova S, Borovikova L, Manogue KR, Faist E, Abraham E, Andersson J, Andersson U, Molina PE, Abumrad NN, Sama A, Tracey KJ. 1999. HMG-1 as a late mediator of endotoxin lethality in mice. Science 285:248–251.

Yang J, Shah R, Robling AG, Templeton E, Yang H, Tracey KJ, Bidwell JP. 2007a. HMGB1 is a bone-active cytokine. J Cell Physiol 214:730–739.

Yang SR, Wright J, Bauter M, Seweryniak K, Kode A, Rahman I. 2007b. Sirtuin regulates cigarette smoke-induced proinflammatory mediator release via RelA/p65 NF-kB in macrophages in vitro and in rat lungs in vivo: Implications for chronic inflammation and aging. Am J Physiol Lung Cell Mol Physiol 292:L567–L576.

Zimmermann K, Völkel D, Pable S, Lindner T, Kramberger F, Bahrami S, Scheiflinger F. 2004. Native versus recombinant high-mobility group B1 proteins: Functional activity in vitro. Inflammation 28:221–229.