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Heme oxygenase-1 protects human periodontal ligament cells against substance P-induced RANKL expression

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Background and Objective: Although substance P (SP) stimulates bone resorption activity and this is reported to be correlated with the degree of periodontal inflammation, it is unclear how human periodontal ligament cells regulate neuropeptide-induced osteoclastogenesis or the possible involvement of heme oxygenase-1 (HO-1) might be. This study examines how SP affects osteoprotegerin (OPG) and RANKL expression via HO-1.

Material and Methods: Using immortalized human periodontal ligament cells, the effects of SP on the expression of HO-1, RANKL and OPG mRNA and proteins were determined by RT-PCR and western blotting, respectively. Various concentrations of SP (10^{-7}, 10^{-8}, 10^{-9} and 10^{-10} M) were added to the medium, and the cells were treated for 0, 0.25, 0.5, 1, 2 and 3 d.

Results: Substance P upregulated RANKL and HO-1 and downregulated OPG mRNA and protein expression in periodontal ligament cells, in a concentration- and time-dependent manner. A HO-1 inducer inhibited both the upregulation of RANKL expression and downregulation of OPG expression by SP in periodontal ligament cells. By contrast, treatment with a HO-1 inhibitor or HO-1 small interferring RNA (siRNA) enhanced SP-stimulated RANKL expression. Inhibitors of ERK and p38 MAP kinases, phosphoinositide 3-kinase and nuclear factor-κB blocked the effects of SP on RANKL expression in periodontal ligament cells.

Conclusion: These results suggest that SP stimulates osteoclastic differentiation by increasing the expression of RANKL vs. OPG via the HO-1 pathway in periodontal ligament cells. The HO-1 pathway may be an effective therapeutic target for inhibiting chronic periodontitis involving alveolar bone resorption.

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Periodontal diseases, including gingivitis and periodontitis, are serious infections and chronic, multifactorial, inflammatory diseases in the oral cavity, which can lead to tooth loss and periodontal bone resorption (1,2). Although chronic pain and bone destruction commonly occur in periodontitis, the relationship between pain (i.e. the potential release of neuropeptide) and bone destruction is not completely understood.

The periodontal ligament is an unmineralized connective tissue that connects the alveolar bone to teeth (3). Periodontal ligament cells have osteoblast-like features and are capable of differentiating into osteoblasts or

cementoblasts (4). In addition, periodontal ligament cells not only support osteoclastogenesis through cell-to-cell contact but also inhibit the formation of tartrate-resistant acid phosphatase-positive multinucleated cells by producing soluble factors (5). Furthermore, periodontal ligament cells express both RANKL and osteoprotegerin (OPG) mRNA (6–8). Given these findings, periodontal ligament cells are involved in regulating alveolar bone metabolism.

Substance P (SP) is a multifunctional neuropeptide that transmits pain signals and regulates the immune system and may modulate emotional stress (9). Substance P is found in nerve fibers of dental pulp and in periodontal tissues of rats, cats, monkeys and humans (10–13). Substance P is also detected in gingival tissues and gingival crevicular fluid, which is correlated with the degree of periodontal inflammation; it has been shown that a reduction in inflammation as a result of effective periodontal treatment is associated with a reduction in the level of SP in gingival crevicular fluid (14,15). Furthermore, Norevall *et al*. (16) reported that the expression of SP was increased after orthodontic tooth movement in rat periodontal ligament specimens, and Sakallioğlu *et al*. (17) found increased SP concentrations in periodontitis sites compared with healthy sites in the mouths of smokers. These observations suggest that SP is involved in remodeling the periodontal ligament and alveolar bone in periodontitis or during orthodontic tooth movement.

Previously, SP was reported to increase bone resorption in an experiment using mouse calvaria (18). Mori *et al*. (18) demonstrated that the addition of SP to cultured rabbit osteoclasts induced an increase in the intracellular calcium concentration, which was abrogated by an SP receptor antagonist. Moreover, it has been demonstrated that SP upregulates osteoclastogenesis by activating RANKL in human dental pulp (19), osteoclast precursors (20) and synovial fibroblastic cells (21).

Heme oxygenase (HO) is a rate-limiting enzyme involved in the catabolism of heme (22). One of the three mammalian HO isoforms, HO-1 (also called heat shock protein 32), is a stress-response protein activated by various agents and is involved in a variety of regulatory and protective mechanisms in cells (23). Recently, we identified the HO-1 pathway as a key mechanism underlying the biological activities of human dental pulp and periodontal ligament cells (24–26). We also found that SP induces the defense protein HO-1 and macrophage inflammatory protein 3α/CCL20 in human periodontal ligament cells (27). In addition, a recent study demonstrated that SP decreased compression-induced RANKL mRNA expression in periodontal ligament cells (28).

Therefore, we hypothesized that HO-1 protects human periodontal ligament cells against SP-induced RANKL expression, which is associated with the degree of periodontal inflammation. This study investigated a role for HO-1 induction in cultured human periodontal ligament cells and the corresponding cytoprotective effects against SP-induced RANKL vs. OPG expression.

Material and methods

Reagents

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and other tissue culture reagents were obtained from Gibco BRL Co. (Grand Island, NY, USA). The HO-1 activator hemin and HO-1 inhibitor zinc protoporphyrin IX (ZnPP IX) were purchased from Porphyrin Products (Logan, UT, USA). Anti-RANKL, anti-OPG and anti-HO-1 antibodies were purchased from Santa Cruz Biotechnology (Heidelberg, Germany). Substance P and all other chemicals were obtained from Sigma Chemical Co. (St Louis, MO, USA) unless otherwise indicated.

Cell culture and treatment

Our previously described HPV16-immortalized human periodontal ligament cells were used (29). Cells were cultured in DMEM (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% FBS (Gibco), 100 U/mL penicillin and 100 μg/mL streptomycin (Bio Whittaker, Walkersville, MD, USA) in a humidified atmosphere of air containing 5% CO_2 at 37°C. The subances selected for investigation in immortalized human periodontal ligament cells were SP (10^{-7}, 10^{-8}, 10^{-9} and 10^{-10} M), hemin (20 μM), ZnPP (20 μM), SB203580 (20 μM), PD98059 (20 μM), LY294002 (20 μM; Calbiochem, LaJolla, CA, USA), MG132 (20 μM) and SP600125 (20 μM).

Isolation of RNA and RT-PCR analysis

Reverse transcription-polymerase chain reaction was performed to analyse RANKL and OPG mRNA expression as described previously (6,23). In brief, total RNA was isolated with TRIzol® reagent from 2×10^6 cells following the manufacturer's (Invitrogen Life Technology, Carlsbad, CA, USA) instructions. For RT, 1 μg of total RNA was used to synthesize cDNA with oligo(dT)18 primer and 200 units of SuperScriptII (Gibco BRL, Rockville, MD, USA). The cDNA was amplified in a final volume of 20 μL containing 2.5 mM magnesium dicholoride, 1.25 units Ex Taq polymerase (AccuPower PCR PreMix; Bioneer, Daejeon, Korea) and 1 μM specific primers. The sequences of the specific primers used in this study were as follows: OPG (6), 324 bp, forward 5′-GCTAACCT-CACCTTC GAG-3′, reverse 5′-TGATTGGACCT GGTTACC-3′; RANKL (6), 486 bp, forward 5′-GCCAGTGGGAGATG TTAG-3′, reverse 5′-TTAGCTGCAA GTTTTCCC-3′; glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 23), 306 bp, forward 5′-CGGAGTCAA CGGATTTGGTCGTAT-3′, reverse 5′-AGCCTTCTCCATGGTGGTG AAGAC-3′; and HO-1 (30), 399 bp, forward 5′- AAGATTGCCCAGA AAGCCCTGGAC-3′, reverse 5′-AA CTGTGCCACCAGAAAGCTGA G-3′. All PCR products were analyzed by 1% agarose gel electrophoresis and visualized by staining the gel

with vvethidium bromide. The intensity of each band after normalization with GAPDH mRNA was quantified on the photographed gels with a densitometer (Quantity One; Bio-Rad, Hercules, CA, USA).

Real-time polymerase chain reaction analysis

Real-time PCR was performed on cDNA samples using the SYBR Green system (Bio-Rad, Richmond, CA, USA). Primers used were as follows: OPG sense 5′-TCCTGGAGCTTTCT GCACAC-3′, OPG anti-sense 5′-AC AGGAAGTATCGCCTGCCT-3′; and RANKL sense 5′-GACACG GTCCCACCATGTTA-3′, RANKL anti-sense 5′-AACACACACTGGGA AAGCCA-3′. Cycling conditions were one cycle at 50°C for 2 min, one cycle at 95°C for 10 min and 40 cycles each of 15 s at 95°C and 1 min at 60°C. Analysis used the sequence detection software supplied with the instrument. The relative quantification value is expressed as $2 \pm \Delta Ct$, where ΔCt is the difference between the mean Ct value of duplicates of the sample and of the GAPDH control.

Preparation of cytosolic and nuclear fractions

Cells were harvested, washed with ice-cold phosphate-buffered saline and kept on ice for 1 min. The suspension was mixed with buffer A (10 mM Hepes, pH 7.5, 10 mM KCl, 0.1 mM EGTA, 0.1 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 5 mg/mL aprotinin, 5 mg/mL pepstatin and 10 mg/mL leupeptin) and lysed by three freeze–thaw cycles. Cytosolic fractions were obtained after centrifugation at 12,000 *g* for 20 min at 4°C. The pellets were resuspended in buffer C (20 mM Hepes, pH 7.5, 0.4 M NaCl, 1 mM EGTA, 1mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 5 mg/mL aprotinin, 5 mg/mL pepstatin and 10 mg/mL leupeptin), kept on ice for 40 min and then centrifuged at 14,000 *g* for 20 min at 4°C. The resulting supernatant was used as soluble nuclear fractions for further experiments.

Western blot analysis for RANKL, OPG, HO-1 and NF-E2-related factor 2 (Nrf-2)

Protein samples (30 μg) were mixed with an equal volume of 2 × SDS sample buffer, boiled for 5 min, and then separated through 8–15% SDS-PAGE gels. After electrophoresis, proteins were transferred to nylon membranes by electrophoretic transfer. The membranes were blocked in 5% skimmed milk (1 h), rinsed, and incubated with anti-HO-1, anti-OPG, anti-RANKL and anti-Nrf-2 antibodies (diluted at 1:1000) in Tris-buffered saline (TBS) overnight at 4°C. Primary antibody was then removed by washing the membranes four times in TBS, and labeled by incubating with 0.1 mg/mL peroxidase-labeled secondary antibodies (against mouse and rabbit) for 1 h. Following three washes in TBS, bands were visualized by enhanced chemiluminescence (ECL) and exposed to X-ray film. The density of the bands after normalization with beta actin were computer-analyzed by densitometer (Quantity One; Bio-Rad).

Transfection of small interferring RNA (siRNA)

The siRNAs were synthesized in duplex and purified forms using Bioneer technology (Daejon, South Korea). The sense and antisense strands of the human HO-1 siRNA are as follows: sense 5′-CCAGUGCCAAGUUCAA-3′ and antisense 5′-UUGAACUU GGUG-CACUGG-3′. The siRNAs were transfected into the periodontal ligament cells using Lipofectamine™ RNAi-MAX (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Silencer Negative Control siRNA (Invitrogen) was used as a negative control and was introduced into the cells using the same protocol. After transfection, cells were cultured in six-well plates at 37°C until needed.

Statistical analysis

Values were calculated as the means and SD. Statistical significance was evaluated by one-way analysis of variance (ANOVA) using the SPSS computer program (v10.0; SPSS, Chicago, IL, USA).

Results

Effects of SP on expression of OPG, RANKL and HO-1 in periodontal ligament cells

Given that the balance between RANKL and its decoy receptor OPG is believed to be critical for regulating osteoclast differentiation and function (6), we examined the effects of SP on RANKL and OPG mRNA and protein expression induced by SP in cultured human periodontal ligament cells. In this study, various concentrations of SP (10^{-7}, 10^{-8}, 10^{-9} and 10^{-10} M) were added to the medium, and the cells were treated for 0.25, 0.5, 1, 2 or 3 d. Very little RANKL mRNA and protein expression was detected in unstimulated periodontal ligament cells. Substance P increased RANKL protein and mRNA expression in a dose- and time-dependent manner, with maximal RANKL induction occurring after 3 d of exposure to 0.1 μM (10^{-7} M) SP. The greatest OPG mRNA and protein expression was detected in unstimulated periodontal ligament cells. Substance P decreased OPG mRNA and protein expression in a dose- and time-dependent manner (Fig. 1).

To examine the effects of SP on the cellular antioxidant defense enzyme HO-1, periodontal ligament cells were treated with different concentrations of SP for 3 d (Fig. 1A) and with 10^{-7} M SP for different times (Fig. 1B). Reverse transcription-polymerase chain reaction and western blot analysis demonstrated that HO-1 induction was dependent on the SP concentration and time. Collectively, SP induced RANKL OPG mRNA and protein expression and HO-1 protein expression and reduced OPG expression in periodontal ligament cells (Fig. 1).

Effects of HO-1 inducer and inhibitor on SP-induced OPG/RANKL and HO-1 expressions in periodontal ligament cells

To further assess the role of HO-1 in periodontal ligament, cells were

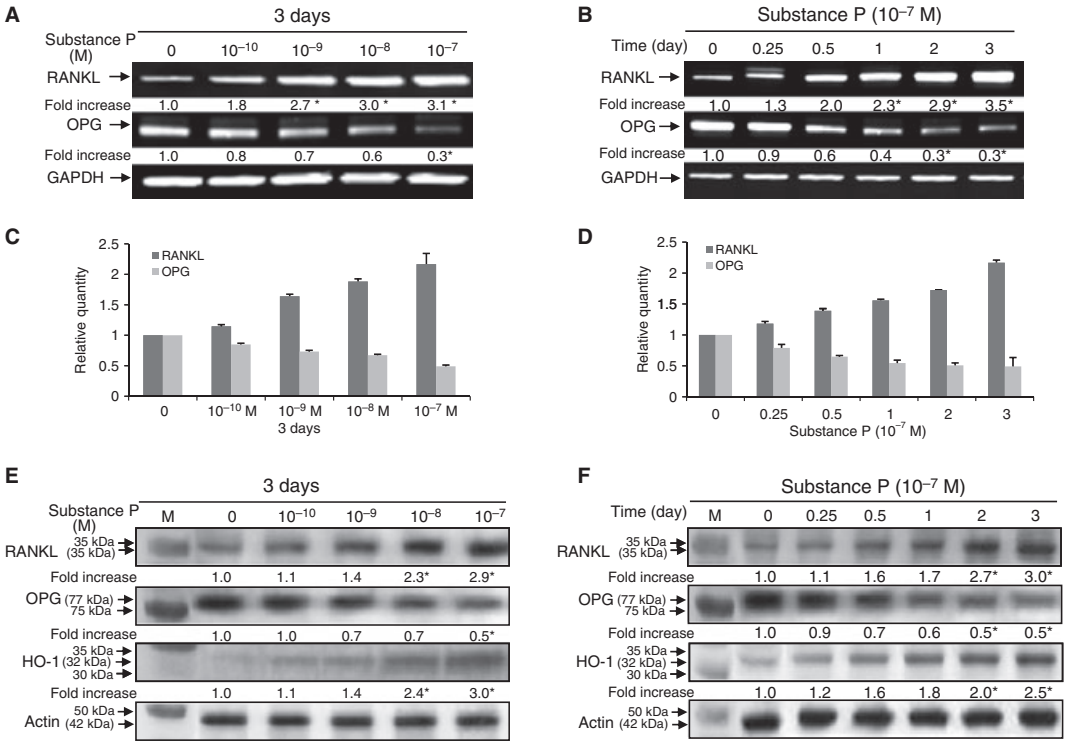

Fig. 1. Effect of substance P (SP) on the expression of RANKL, osteoprotegerin (OPG) and heme oxygenase-1 (HO-1) mRNA and protein. Western blot analysis (E, F), real-time PCR (C, D) and RT-PCR (A, B) for RANKL, OPG and HO-1 expression were performed as described under Materials and methods. Numbers below the gels or bands represent the intensity of RANKL, OPG and HO-1 mRNA or protein relative to GAPDH mRNA or β-actin. Results are representative of three independent experiments. *Statistically significant difference compared with control group, $p < 0.05$.

pretreated with hemin (a potent HO-1 inducer) or zinc protoporphyrin IX (ZnPPIX, a potent HO-1 inhibitor) to enhance or decrease the induction of RANKL and OPG by SP. As shown in Fig. 2, HO-1 expression was enhanced by hemin, and this resulted in a significant reduction in the SP-induced effect on RANKL expression, while restoring the SP-induced effect on OPG downregulation. Conversely, ZnPPIX pretreatment blocked the induction of HO-1 expression by SP, while enhancing the RANKL-inducing effects of SP.

Roles of MAPK, phosphoinositide 3-kinase (PI3K) and nuclear factor-κB (NF-κB) on SP-induced RANKL/OPG and HO-1 expression in human periodontal ligament cells

Previous studies have demonstrated that activation of the mitogen-activated protein kinase (MAPK) and PI3K pathways and NF-κB contrib-

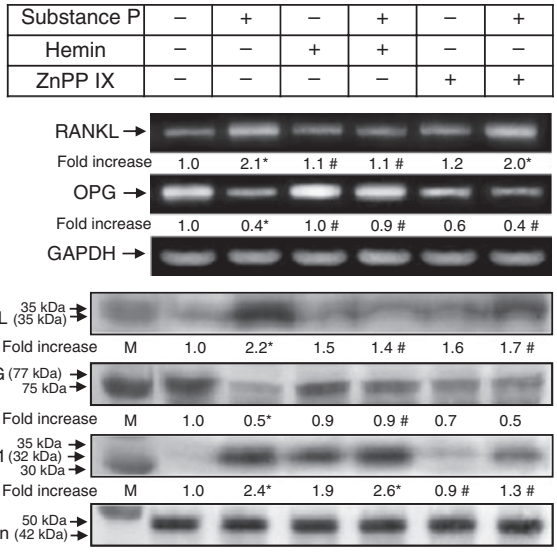

Fig. 2. Effects of HO-1 inducer (hemin) and inhibitor (ZnPP) on SP-mediated RANKL, OPG and HO-1 expression. Periodontal ligament cells were pretreated with hemin (20 μM) or ZnPP (20 μM) for 16 h, and treated for 3 d with SP (10^{-7} M). Results are representative of three independent experiments. *Statistically significant difference compared to control group, $p < 0.05$; #statistically significant difference compared with SP-treated group, $p < 0.05$.

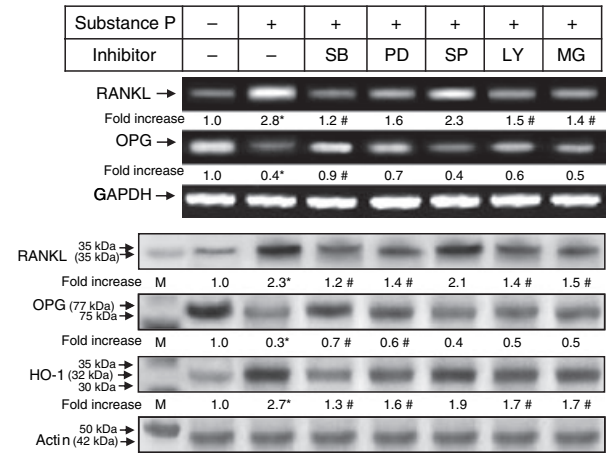

Fig. 3. Effects of various signal pathway inhibitors on SP-induced RANKL, OPG and HO-1 expression in human periodontal ligament cells. Cells were pretreated with various key signal pathway inhibitors (PI3K inhibitor LY294002, ERK1/2 inhibitor PD98059, p38 MAPK inhibitor SB203580, JNK inhibitor SP600125 or NF-κB inhibitor MG132) and then incubated with 10^{-7} M of SP. Results are representative of three independent experiments. *Statistically significant difference compared with control group, $p < 0.05$; #statistically significant difference compared with SP-treated group, $p < 0.05$.

utes to the induction of HO-1 (31). In addition, we previously reported that SP induces NF-κB, p38 and ERK1/2 MAPK activation in periodontal ligament cells (27). To examine the signaling pathways involved in the SP-induced RANKL/OPG and HO-1 expression, periodontal ligament cells were pretreated with various inhibitors of key signaling molecules (Fig. 3). In periodontal ligament cells, SP-induced RANKL and HO-1 expression was inhibited by the p38 inhibitor SB203580 (20 μM), the selective ERK1/2 pathway inhibitor PD98059 (20 μM), the PI3 kinase inhibitor LY294002 (20 μM) and the specific membrane-permeable proteasome inhibitor MG132 (20 μM), but not by the JNK inhibitor SP600125 (20 μM; Fig. 3).

Effects of SP on Nrf-2 nuclear translocation in periodontal ligament cells

Nuclear translocation of activated Nrf-2 is an important upstream step in the mechanism of HO-1 expression. The Nrf-2 signaling pathway regulates the expression of HO-1 in several cell types (32). However, the same type of regulation system has not been reported in periodontal ligament cells. We examined Nrf-2 expression and nuclear translocation in periodontal ligament cells. As evidenced by western blots using the nuclear fraction, compared with the control, SP treatment induced translocation of Nrf-2 in a dose-dependent manner, while the level of Nrf-2 in the cytoplasm decreased (Fig. 4).

Effect of silencing HO-1 with siRNA on SP-induced RANKL and OPG expression

Periodontal ligament cells were transfected with HO-1 siRNA, and the expression of RANKL and OPG was measured to determine whether HO-1 is directly responsible for RANKL and OPG expression. Transfection with HO-1 siRNA resulted in the targeted knockdown of HO-1 mRNA expression, whereas the same amount of nonspecific siRNA had no inhibitory effect on HO-1 expression in periodontal ligament cells (Fig. 5). The HO-1 siRNA enhanced the effects of SP on RANKL and OPG expression.

Discussion

Substance P is thought to be involved in a range of immune responses and may be a chronic mediator of bone metabolism (9,10). Although SP and its receptors are located in periodontal tissue (11–16,28), their roles in periodontitis and bone remodeling during orthodontic tooth movements are still poorly understood. In this study, the action of SP was observed at concentrations of 10^{-7}–10^{-9} M; this concentration range is similar to the SP concentration in gingival crevicular fluid (0.1 μM, 10^{-7} M) in periodontitis patients, suggesting that SP promotes the differentiation of osteoclasts in periodontal tissue with periodontitis (15). We previously reported that immortalized periodontal ligament cells have high alkaline phosphatase activity, calcified nodule formation, gene expression patterns and osteogenic differentiation patterns in culture that are similar to those of primary

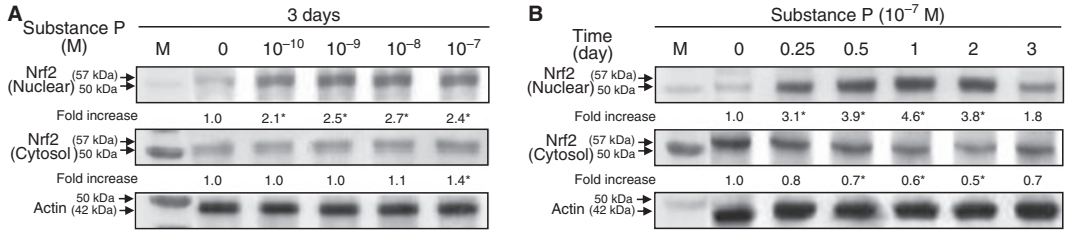

Fig. 4. Accumulation and distribution of Nrf-2 protein level after treatment of periodontal ligament cells with SP. Cells were treated for 3 d with the indicated concentrations of SP. Results are representative of three independent experiments. *Statistically significant difference compared with control group, $p < 0.05$; #statistically significant difference compared with SP-treated group, $p < 0.05$.

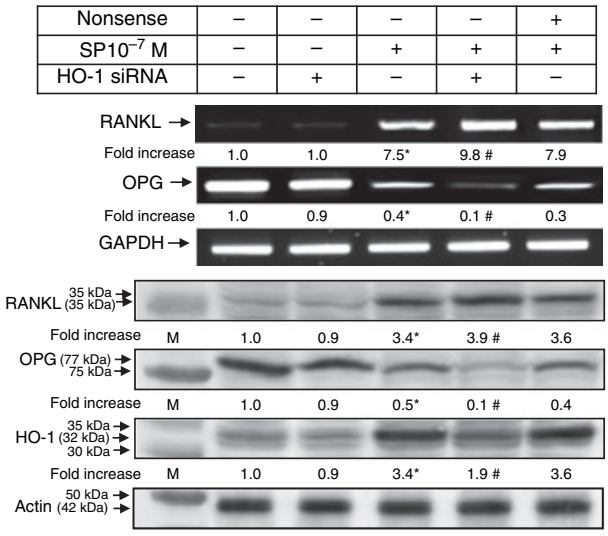

Fig. 5. Roles of HO-1 in inhibition of SP-induced RANKL and OPG expression. Cells were first transfected with control siRNA or HO-1 siRNA and thereafter treated with SP for 3 d. Results shown are representative of three independent experiments. *Statistically significant difference compared with control group, $p < 0.05$; # statistically significant difference compared with SP-treated group, $p < 0.05$.

cultured periodontal ligament cells (29). Thus, we postulated that immortalized periodontal ligament cells can be used in periodontal research to characterize and elucidate the molecular mechanisms of osteogenic signaling. Since human periodontal ligament cells are crucial in periodontal regeneration and repair, an examination of how SP affects periodontal ligament cells could provide valuable information. Therefore, this study investigated the cellular and molecular cytoprotective defense mechanisms against SP-induced osteoclast differentiation of periodontal ligament cells, focusing on the roles of RANKL, OPG and HO-1.

In periodontal tissues, RANKL and OPG are important regulators of balanced alveolar bone resorption (6–8). Our results in human periodontal ligament cells indicate that SP downregulated OPG and upregulated RANKL expression (Fig. 1). These results are consistent with those of other studies on the SP-induced effect on RANKL expression in dental pulp (19), osteoclast precursors (20), synovial fibroblasts (21) and periodontal ligament cells (28). Our findings suggest that continuous SP secretion by sensory neurons in painful chronic periodontitis or tooth movement is likely to increase RANKL expression and decrease OPG expression in periodontal ligament cells.

Heme oxygenase-1 plays an important role in cytopvvrotection against pathological conditions, including arthritis (33). Although we previously demonstrated that SP can induce HO-1 in periodontal ligament cells (27), there has been no report on the involvement of HO-1 in a defense mechanism against SP-induced osteoclastic differentiation in periodontal ligament cells. Here, we report that SP increased the expression of RANKL and HO-1 in periodontal ligament cells.

Next, we investigated whether the induction of HO-1 expression affected the SP-mediated RANKL upregulation and OPG downregulation in periodontal ligament cells. Pre-incubation of periodontal ligament cells with the HO activator, hemin, resulted in protection against the effects of SP on RANKL and OPG expression (Fig. 2). Conversely, the specific HO competitive inhibitor ZnPPIX and HO-1 siRNA enhanced SP-mediated RANKL expression (Figs 2 and 5). According to Zwerina *et al.* (22), the HO-1 inducer, hemin, caused dose-dependent upregulation of HO-1 in osteoclast precursors and inhibited osteoclast differentiation, consequently inhibiting bone resorption (22). Moreover, our data suggest that the HO-1 pathway has a beneficial effect on periodontitis by modulating RANKL expression. Considering that RANKL is critical for osteoclast differentiation, the effect of HO-1 on osteoclastogenesis in human periodontal ligament cells should be studied further with regard to periodontal bone resorption and destruction.

The upstream signaling mechanism responsible for regulating HO-1 transcription is poorly defined. The MAPKs are believed to play a significant role in regulating the inflammatory response and differentiation in osteoclasts and periodontal ligament cells (34). However, the contribution of each individual pathway is rather difficult to define because complex interactions among all of the involved pathways directly or indirectly affect the final outcome. The MAPK families can be classified as stress-activated kinases (p38/JNK) or extracellular signal-regulated kinases (ERK). In addition, NF-κB and PI3K are crucial in cell survival signaling and the modulation of HO-1 expression (35). Recent studies have demonstrated the involvement of the p38, JNK, ERK, PI3K and NF-κB pathways in the HO-1 response to stress (36). In our study, the use of specific inhibitors of the MAPK, PI3K and NF-κB pathways confirmed the involvement of the ERK, p38, Akt and NF-κB pathways, but not the JNK pathway, in SP-induced HO-1 expression and osteoclastic differentiation (Fig. 5).

Nuclear respiratory factor-2, a relatively well-known transcription factor for HO-1, has been implicated in stress-induced HO-1 expression (22). In unstimulated cells, Nrf-2 resides in the cytoplasm, bound to the actin-binding protein Kelch-like ECH-associated protein 1 (Keap1). When cells are activated by stimulation, such as oxidative stress, Nrf-2 separates from Keap1 and translocates into the nucleus, where it binds to the antioxidant response element of the HO-1 promoter (32). In our study, SP also induced the translocation of Nrf-2 into the nucleus, as evidenced by western

blots (Fig. 4). Furthermore, the SP-induced effects on OPG and RANKL expression were dependent on the HO-1 pathway (Fig. 5). These results suggest that the nuclear translocation of Nrf-2 plays a key role in SP-induced HO-1 expression and cytoprotection.

In summary, this is the first report showing that SP-induced nuclear translocation of Nrf-2 increases HO-1 mRNA and protein expression and thereby increases the RANKL/OPG expression ratio, via ERK, p38, PI3K/Akt and NF-κB activation. Therefore, SP-induced HO-1 signaling is a potential therapeutic target to prevent emotional stress-induced activation of osteoclastic bone destruction in periodontitis.

Acknowledgements

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