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Induction of IL-8 in Periodontal Ligament Cells by H_2O_2

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Periodontitis is an inflammatory disease caused by bacteria. In periodontitis, reactive oxygen species (ROS) are released from inflammatory cells in response to bacteria. Interleukin (IL)-8 is one of pro-inflammatory cytokines. To investigate the role of ROS in pathogenesis of periodontitis, we estimated the effect of H_2O_2, one of ROS, on the expression of IL-8 in human periodontal ligament (PDL) cells. PDL cells were treated with H_2O_2. IL-8 expression was determined by reverse transcription-polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA). The phosphorylation of extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (p38) and c-jun NH_2-terminal kinase (JNK) was estimated by Western blotting. Treatment with H_2O_2 at concentration of up to 250 μM increased IL-8 mRNA expression and production in a concentration-dependent manner. However, treatment with 500 μM H_2O_2 did not increase IL-8 production. Catalase, an inhibitor of H_2O_2, down-regulated the production of IL-8 induced by H_2O_2. H_2O_2 increased the phosphorylation of ERK, p38, and JNK. Pretreatment with PD98059 (ERK inhibitor), SB203580 (p38 inhibitor), or SP600125 (JNK inhibitor) decreased the IL-8 production induced by H_2O_2. These results indicate that H_2O_2 acts as an inducer of IL-8 secretion via activation of ERK, p38, and JNK in PDL cells. H_2O_2 deposited in periodontal tissue during inflammation against bacteria may accelerate tissue destruction via induction of IL-8 in PDL cells.

***Keywords*:** IL-8, periodontal ligament cells, H_2O_2

Superoxide anion, which is formed by NADPH oxidase, xanthin oxidase or mitochondrial electron transport chain (Droge, 2002), is converted into hydrogen peroxide (H_2O_2) and singlet oxygen. These reduced metabolites of O_2 are referred to as "reactive oxygen species (ROS)" (Thannickal and Fanburg, 2000; Dröge, 2002). ROS cause damage to lipid, protein, and DNA, and this effect acts harmful or beneficial to host. ROS function as a tissue destructive factor by damaging components of host cells (Valko *et al.*, 2006, 2007). In contrast, ROS function as an antibacterial factor by damaging components of pathogens within phagocytes (Valko *et al*., 2006, 2007). Recently, ROS have also been recognized as signaling molecules that regulate cell proliferation and the expression of genes (Genestra, 2007).

Periodontitis is a bacteria-induced inflammatory disease that is accompanied by gingival inflammation and alveolar bone loss. The tissue damage induced by periodontitis is caused directly by bacteria and indirectly by inflammation and immune response against bacteria (Ishikawa, 2007). In the inflammatory process, activated phagocytes such as neutrophils and macrophages produce large quantities of ROS via the NADPH oxidase (Valko *et al*., 2007). Patients with periodontal disease display increased neutrophil number and activity (Kimura *et al.*, 1993; Liu *et al.*, 2001; Sculley and Langley-Evans, 2002). *Fusobacterium nucleatum*, a periodontopathogen, increases not only the intracellular production of ROS, but also the extracellular release of ROS in neutrophils (Sheikhi *et al.*, 2000, 2001). In saliva or gingival crevicular fluid of periodontitis patients, level of lipid peroxidation induced by ROS was higher than that of healthy controls (Tsai *et al.*, 2005). In addition, it has been demonstrated that hydroxyl radicals induce major oxidative damage to gingiva *in vitro* (Bartold *et al.*, 1984). Furthermore, radical scavenger has been found to decrease all parameters associated with inflammation in rat periodontitis, including myeloperoxidase activity, vascular permeability, and bone destruction, suggesting that antioxidant treatment, which interferes with ROS, is beneficial for the treatment of periodontitis (Di Paola *et al.*, 2005). Taken together, periodontopathogens stimulate neutrophils to release ROS, which then cause damage to periodontal tissue. Although it has been suggested that ROS are important in the pathogenesis of periodontitis, very little is known about the exact role of ROS in tissue destruction of periodontitis. A better understanding of the effects of ROS on cells of periodontal tissue could provide important information regarding the pathogenesis of periodontitis.

Cytokines, which induce inflammation and bone resorption, play an important role in the pathogenesis of periodontitis. Interleukin (IL)-8 is a CXC chemokine that functions as pro-inflammatory cytokine. IL-8 is a potent neutrophil chemoattractant and activator (Baggiolini and Clark-Lewis, 1992). Receptor activator of NF-κB ligand (RANKL), a tumor necrosis factor (TNF) family cytokine, induces osteo-

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clast formation and palys an important role in bone resorption (Tanaka, 2007). It has been reported that IL-8 stimulated osteoclast formation either in the presence or absence of RANKL (Bendre *et al.*, 2003, 2005). In periodontitis, the level of IL-8 in gingival crevicular fluid and periodontal tissue is increased, which is correlated with disease activity (Tsai *et al.*, 1995; Gamonal *et al.*, 2000, 2001). Therefore, IL-8 may be involved in pathogenesis of periodontitis as an inflammation and bone resorption-inducing factor. Periodontal ligament (PDL) cells, between alveolar bone and tooth root, are one of representative cells in periodontal tissue. In this study, we examined the effect of H_2O_2 on the expression of IL-8 and the mechanism that is required for IL-8 induction by H_2O_2 in PDL cells.

Materials and Methods

Primary cultures of periodontal ligament (PDL) cells

PDL tissues were obtained from extracted teeth for orthodontic treatment. The protocol was approved by Institutional Review board of Yonsei Dental Hospital. Primary human PDL cells were cultivated from PDL tissues attached to the middle one third of the extracted premolar and molar tooth roots. Using blades, PDL tissue fragments were removed from tooth and were washed three times with α-modified Eagle's medium (α-MEM, Gibco BRL, USA) containing antibiotics of 300 mg/ml streptomycin, 0.75 mg/ml amphotericin-B, and 300 unit/ml penicillin (Gibco BRL, USA). PDL tissue fragments were placed in 100-mm dishes containing α-MEM supplemented with antibiotics of 100 mg/ml streptomycin, 0.25 mg/ml amphotericin-B, 100 unit/ml penicillin, and 20% fetal bovine serum (FBS, Gibco BRL, USA). The cultures were replaced every 2 days until cells grew form the fragments, after which the cultures were maintained with α-MEM containing antibiotics and 10% FBS. Upon reaching 70% confluence, the cells were removed with 0.25% trypsin and passaged to 100-mm dishes. The cells used in this study had been passaged 5 to 8 times.

Treatment of H_2O_2 in PDL cells

PDL cells were seeded at 5×10^5/well in 6-well plates and grown in α-MEM supplemented with antibiotics and 10% FBS. After reaching confluency, the cells were incubated with H_2O_2 (Calbiochem, USA) at the indicated concentrations for 8 h. For pretreatment, the cells were incubated with catalase (Sigma Chemical Co., USA) or each mitogen-activated protein kinases (MAPKs) inhibitor (PD98059, SB203580 or SP600125, Sigma Chemical Co.) for 30 min and then treated with H_2O_2 for 8 h. After the H_2O_2 treatment, the cells were processed for Western blotting or reverse transcription-polymerase chain reaction (RT-PCR) and the culture supernatants were processed for enzyme-linked immunosorbent assay (ELISA).

Reverse transcription-PCR (RT-PCR)

Gene expression of IL-8 and glyceraldehyde-3-phosphatedehydrogenase (GAPDH) was analyzed using RT-PCR. Total RNA was extracted using Trizol reagent (Invitrogen Corp., USA) and 1 μg of total RNA was then converted to cDNA using an RT Premix Kit (Bioneer, Korea) according to the manufacturer's instructions. PCR was performed using a PCR Premix Kit (Intron Biotechnology, Korea). The primers (25 pM) used for PCR were as follows: IL-8 sense primer, 5'-ATGACTTCCAAGCTGGCCGTGGCT-3'; antisense primer, 5'-TCTCAGCCCTCTTCAAAAACTTCTC-3'; GAPDH sense primer, 5'-CGGAGTCAACGGATTTGGTCGTAT-3'; and antisense primer 5'-AGCCTTCTCCATGGTGGTGAA GAC-3'. For IL-8, the reaction mixtures were subjected to 25 cycles of denaturation at 95°C for 30 sec, annealing at 62°C for 30 sec, and extension at 72°C for 30 sec. For GAPDH, the reaction mixtures were subjected to 27 cycles consisted of denaturation at 95°C for 30 sec, annealing at 56°C for 30 sec, and extension at 72°C for 30 sec. The RT-PCR products were then resolved by electrophoresis in 1% agarose gel containing ethidium bromide and digitally photo-graphed using Chemiimager (Alpha Innotech, USA). All assays were performed in duplicate in three separate experiments.

ELISA

The level of IL-8 in culture supernatants of PDL cells was quantitatively assayed using an ELISA kit (R & D systems, USA) with recombinant IL-8 as a standard in accordance with the manufacturer's protocol. All assays were performed in duplicate in three separate experiments.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay

Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. PDL cells (1.5×10^5 cells/well) were seeded in 24-well plates and then grown for 20 h. Next, the cells were treated with the indicated concentrations of H_2O_2 for 8 h. After treatment, the plates were centrifuged at 200×g for 10 min and the medium was then replaced with fresh medium. Twenty microliter of MTT solution (5 mg/ml in PBS, Sigma Chemical Co.) was added to each well. After incubation for 4 h, the plates were centrifuged, aspirated the medium, and 300 μl of dimethylsulfoxide (DMSO) was added to each well to dissolve MTT-formazan crystals. The plates were shaken for 5 min and the absorbance was measured on an MRX II microplate reader (Dynatech Laboratories, USA) at 570 nm. All assay were performed in duplicate in two separate experiments

Western blot analysis

The phosphorylation of MAPKs, including extracellular signal-regulated kinase (ERK), p38 MAPK (p38), and c-Jun N-terminal kinase (JNK), in PDL cells treated with H_2O_2 was measured by Western blotting. Following incubation with H_2O_2 for the indicated times, the cells were placed on ice, rinsed twice with ice-cold phosphate-buffered saline and then lysed with lysis buffer containing a protease inhibitor cocktail (Roche, Germany). The cell lysates were then centrifuged at 12,000×g at 4°C, after which the protein concentrations were determined using the protein assay reagent (Bio-Rad Laboratories, Inc., USA). Equivalent amounts of total protein per sample of cell extract were run on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immediately transferred to a poly(vinylidene di-

fluoride) (PVDF) membrane. The membranes were blocked with 3% bovine serum albumin in tris-buffered saline (TBS) containing 0.1% Tween 20 for 1 h, rinsed, and then incubated overnight with the primary antibodies against phospho-ERK, phospho-p38, phospho-JNK, ERK, p38 or JNK (Cell Signaling Technology, USA) at a dilution of 1:1000. The membranes were then washed four times, after which they were incubated with a secondary peroxidase-conjugated anti-rabbit immunoglobulin (Santa Cruz Biotechnology, Inc., USA) at a dilution of 1:2500 for 1 h and then rinsed. Immunopositive bands were visualized using an ECL kit (Amersham Bioscience, Sweden). All assays were performed in duplicate in three separate experiments.

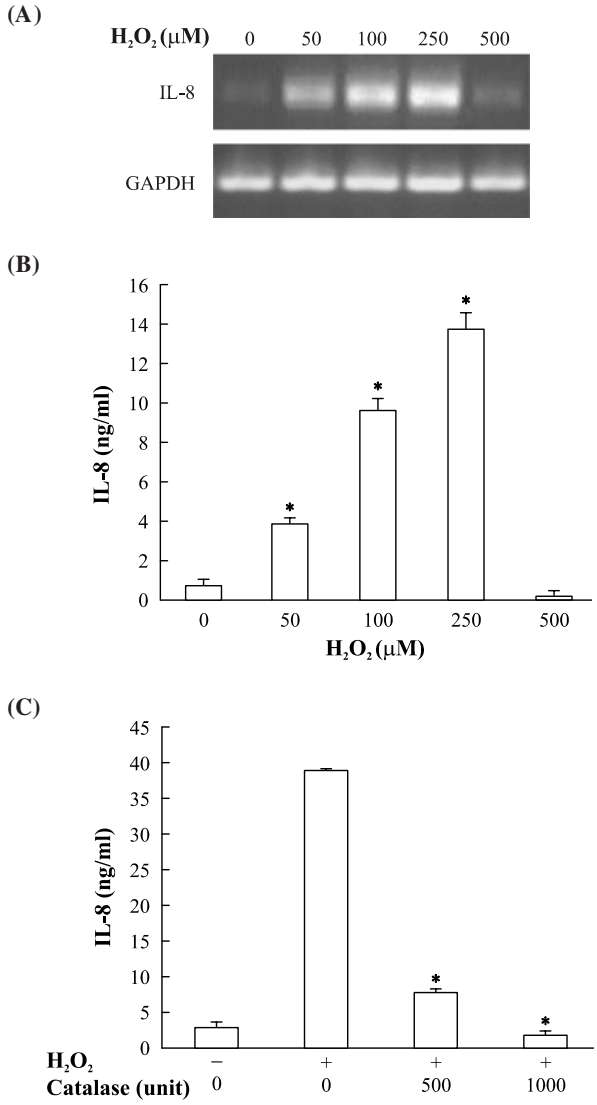

Fig. 1. H_2O_2-induced IL-8 production in PDL cells. PDL cells were treated with H_2O_2 at the indicated concentrations for 8 h. Expression of IL-8 mRNA was estimated by RT-PCR (A) and the concentration of IL-8 in the culture supernatant was determined by ELISA (B). *Significantly different from non-treated cells ($P<0.05$). PDL cells were pretreated with catalase at the indicated concentrations for 30 min followed by exposure to 250 μM H_2O_2. The concentration of IL-8 in culture supernatant was determined by ELISA (C). *Significantly different from H_2O_2-treated catalase-free cells ($P<0.05$).

Statistical analysis

The results were analyzed using one-way ANOVA and Tukey's test. A P value of <0.05 was considered to indicate statistical significance.

Results

Effect of H_2O_2 on IL-8 production

To determine the induction of IL-8 production in PDL cells by H_2O_2, PDL cells were treated with the indicated concentrations of H_2O_2 for 8 h and the IL-8 expression was evaluated by RT-PCR and ELISA. Treatment with H_2O_2 at concentrations of up to 250 μM increased the expression of IL-8 mRNA in a dose-dependent manner (Fig. 1A). However, treatment with H_2O_2 at a concentration of 500 μM failed to increase the IL-8 mRNA expression. Evaluation of the production of IL-8 in response to treatment H_2O_2 using ELISA and RT-PCR produced similar results (Fig. 1B). When compared with the production of non-treated cells, treatment

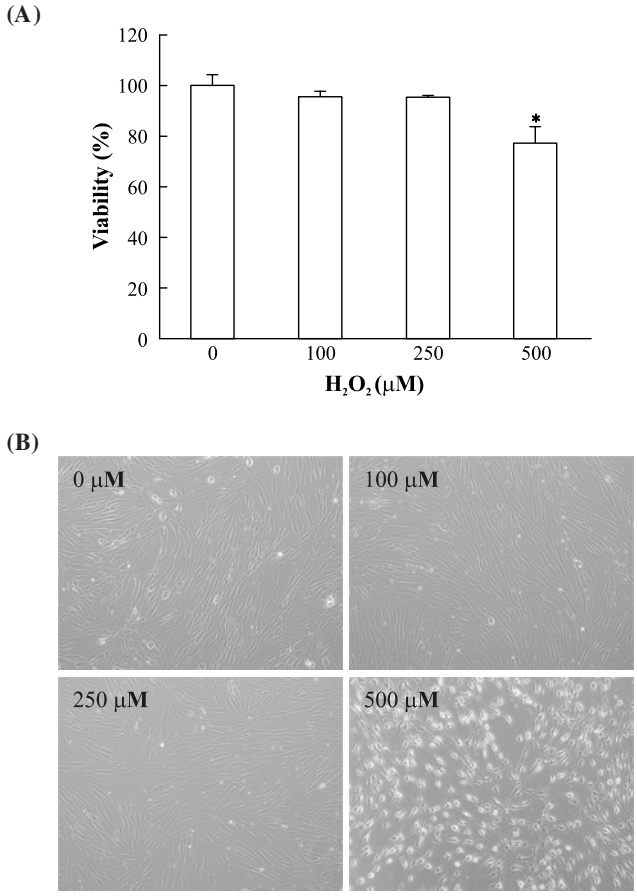

Fig. 2. Effect of H_2O_2 on the viability of PDL cells. PDL cells were treated with H_2O_2 at the indicated concentrations for 8 h and the cell viability was then determined by MTT assay (A). *Significantly different from non-treated cells ($P<0.05$). PDL cells showed morphological changes following treatment with 500 μM H_2O_2 for 8 h (B, ×100).

with H_2O_2 at concentration of 50, 100, and 250 μM increased the IL-8 production to 5-fold, 13-fold, and 19-fold, respectively, with the peak production being observed in response to treatment with 250 μM of H_2O_2. Catalase activates the decomposition of H_2O_2 into water and oxygen and it functions as an antioxidant. When PDL cells were pretreated with catalase, H_2O_2-induced IL-8 production was completely abolished (Fig. 1C). Taken together, these data indicate that H_2O_2 induces the production of IL-8 in PDL cells.

Effect of H_2O_2 on the viability of PDL cells

The effect of H_2O_2 on the viability of PDL cells was evaluated by MTT assay (Fig. 2A). Treatment of cells with H_2O_2 at concentrations of up to 250 μM which induced IL-8 production did not reduce the cell viability. However, treatment of cells with 500 μM H_2O_2 which failed to increase IL-8 production reduced cell viability and caused cell shrinkage (Fig. 2A and B). These results indicate that the failure of 500 μM H_2O_2 to induce IL-8 production occurs due to the cytotoxicity of H_2O_2.

Involvement of the MAPKs pathway in the IL-8 production induced by H_2O_2

To determine whether H_2O_2-induced IL-8 production requires ERK, p38, and JNK pathways, the phosphorylation of ERK, p38, and JNK by H_2O_2 (250 μM) was estimated by Western blotting (Fig. 3). Treatment with H_2O_2 for 30 min and 60 min increased the phosphorylation of ERK, p38, and JNK. To confirm the involvement of the ERK, p38, and JNK pathways in the induction of IL-8 by H_2O_2, PDL cells were pretreated with PD98059 (ERK inhibitor), SB203580 (p38 inhibitor) or SP600125 (JNK inhibitor) prior to exposure to H_2O_2 (250 μM), and the levels of IL-8 protein were detected by ELISA (Fig. 4). The induction of IL-8 by H_2O_2 was reduced by 75% (PD98059, 20 μM), 67% (SB203580, 20 μM) and 76% (SP600125, 20 μM) of the levels induced by H_2O_2 in the absence of each inhibitor. Taken together, these results indicate that ERK, p38, and JNK mediate the induction of IL-8 by H_2O_2.

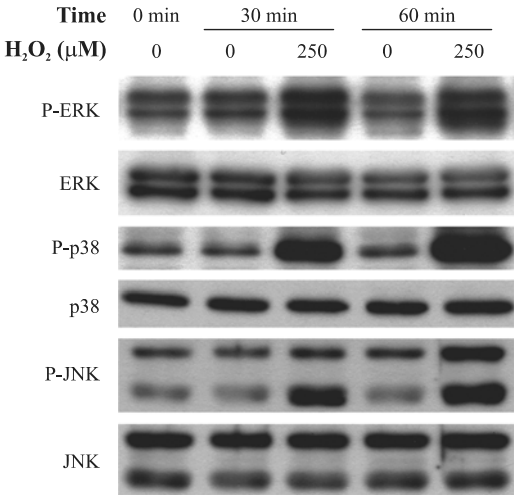

Fig. 3. H_2O_2 -induced phosphorylation of MAPKs in PDL cells. PDL cells were treated with 250 μM H_2O_2 for 30 min and 60 min. The cell lysates were subjected to Western blot analysis with anti-ERK, anti-p38, anti-JNK, anti-phosphorylated ERK, anti-phosphorylated p38 or anti-phosphorylated JNK antibodies. Photograph is representative of the results of three experiments.

Discussion

In periodontitis, ROS are generated by activated phagocytes such as neutrophils and macrophages via NADPH oxidase. ROS, when deposited extracellularly, may destroy tissues,

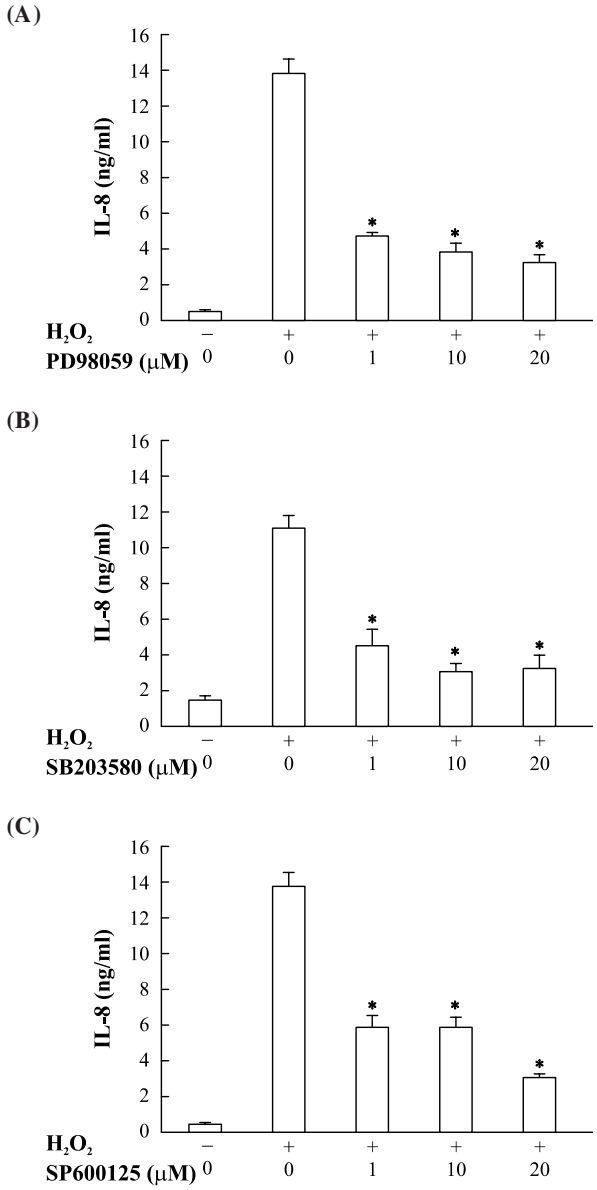

Fig. 4. Effect of each MAPK inhibitor on H_2O_2-induced IL-8 production in PDL cells. PDL cells were pretreated with PD98059 (ERK inhibitor, A), SB203580 (p38 inhibitor, B) or SP600125 (JNK inhibitor, C) for 30 min and then treated with 250 μM H_2O_2 for 8 h. The concentration of IL-8 in culture supernatants was determined by ELISA. *Significantly different from H_2O_2-treated MAPK inhibitor-free cells ($P<0.05$).

directly by damaging the DNA, protein, and lipid of cells and indirectly by inducing cytokines associated with tissue destruction. However, little is known about the indirect effects of ROS in the pathogenesis of periodontitis. Among ROS, H_2O_2 can diffuse freely across the cell membrane (Thannickal and Fanburg, 2000), therefore, we evaluated the effect of ROS on IL-8 induction in PDL cells using H_2O_2. The results of this study indicate for the first time that H_2O_2 induces IL-8 expression in PDL cells and that ERK, p38, and JNK are involved in this process.

Neutrophil accumulation in a tissue is characteristic of inflammation (Kumar *et al.*, 2005). IL-8 functions as a neutrophil chemoattractant and activator by inducing the expression of adhesion molecules (CD11/18), the migration, and the release of enzymes and ROS in neutrophils (Baggiolini *et al.*, 1989). The neutrophil accumulation and activation by IL-8 protects the host by removing pathogens, but produces deleterious effect by destroying tissues. In addition to pro-inflammatory activity of IL-8, it has been reported that IL-8 induces bone resorption by stimulating osteoclast formation both dependent of and independent of RANKL (Bendre *et al*., 2003, 2005). In this study, H_2O_2-treated PDL cells produced at least 6-fold more IL-8 than non-treated cells and catalase, an inhibitor of H_2O_2, abolished H_2O_2-inudced IL-8 expression. These results indicate that H_2O_2 acts as an inducer of IL-8 in PDL cells and this activity of H_2O_2 contributes to periodontal tissue destruction by enhancing neutrophil accumulation and osteoclast formation.

H_2O_2 regulates IL-8 induction in a cell specific manner. It has been shown that H_2O_2 did not induce IL-8 production in umbilical vein endothelial cells and pulmonary artery endothelial cells (DeForge *et al.*, 1993). However, dendritic cells, monocytes, lymphocytes, skin fibroblasts, epithelial cells, and cancer cell lines produce IL-8 in response to treatment with concentrations of H_2O_2 ranging from 25∼4,000 μM (Lakshminarayanan *et al.*, 1997; Verhasselt *et al.*, 1998; Josse *et al.*, 2001; Hwang *et al.*, 2004; Pelaia *et al.*, 2004). In this study, treatment with 50 μM H_2O_2 was found to increase the production of IL-8 by PDL cells, with peak production being observed in response to treatment with 250 μM H_2O_2. However, the viability of cells treated with 500 μM of H_2O_2 decreased and the expression of IL-8 was not induced. These results suggest that, in PDL cells, H_2O_2 acts as an inducer of IL-8 expression at low concentrations and a cytotoxic factor at concentrations higher than 500 μM. Although the concentration of H_2O_2 accumulated in the periodontal tissues of periodontitis patients has not been estimated, H_2O_2 deposited in periodontal tissue by activated phagocytes produces deleterious effect on periodontal tissue by stimulating IL-8 production in PDL cells or by damaging to PDL cells.

In PDL cells, periodontopathogens and pro-inflammatory cytokines also stimulate production of IL-8. Following stimulation of *Porphyromoans gingivalis* or *Prevotella intermedia,* expression of IL-8 in PDL cells was found to increase (Yamamoto *et al.*, 2006). In addition, some components of periodontopathogens such as lipopolysaccharide (LPS) of *P. gingivlais* and the major surface protein of *Treponema maltophilum* and *T. lecithinolyticum* have been found to increase IL-8 expression (Yamaji *et al.* 1995; Lee *et al.*, 2005). Furthermore, pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α and IL-1β have been found to increase mRNA expression of IL-8 (Long *et al.*, 2001; Lee *et al.*, 2006). The experiments conducted in this study did not estimate the combination effect of these factors and H_2O_2. However, it has been shown that exposure of other cells such as colon carcinoma cells and normal intestinal epithelial cells exposure to H_2O_2 followed by treatment with IL-1α, results in enhanced IL-8 production when compared with cells treated with IL-1α alone (Yamamoto *et al.*, 2003), which suggests that H_2O_2 may enhance IL-8 production by periodontopathogens and pro-inflammatory cytokines in cases of periodontitis.

There are three MAPKs: ERK, p38, and JNK. TNF-α has been shown to induce IL-8 secretion via the ERK and p38 in PDL cells (Lee *et al*., 2006). To identify the involvement of MAPKs in H_2O_2-induced IL-8 production in PDL cells, we estimated the phosphorlyation of MAPKs and the inhibitory effect of each MAPK inhibitor on H_2O_2-induced IL-8 production. In PDL cells, H_2O_2 increased the phosphorylation of ERK, p38, and JNK and pretreatment with each inhibitor of ERK (PD98059), p38 (SB203580), and JNK (SP600125) reduced this H_2O_2-induced IL-8 production by 67∼76%. This indicates that, in PDL cells, ERK, p38, and JNK act as signal molecules in H_2O_2-induced IL-8 production. In monocytes, H_2O_2 led to the production of IL-8 and increased phosphorylation of p38, but not ERK. In addition, p38 inhibitor (SB203580) decreased the accumulation of IL-8 mRNA after treatment with H_2O_2, suggesting that p38 is important in the production of IL-8 in H_2O_2-treated monocytes (Josse *et al*., 2001). In bronchial epithelial cells, H_2O_2 increased the phosphorylation of ERK, p38, and JNK, and pretreatment of bronchial epithelial cells with mixtures of p38, ERK, and JNK inhibitors reduced the IL-8 secretion induced by H_2O_2 (Hwang *et al*., 2004), suggesting that these MAPKs act as transducers of IL-8 expression. The findings of these studies suggest that different cells have different signaling pathways that lead to production of IL-8 in response to H_2O_2 and that the signal pathway of IL-8 in PDL cells is similar to that of epithelial cells.

In conclusion, H_2O_2 increased the IL-8 secretion and phosphorylation of ERK, p38, and JNK in PDL cells. In addition, inhibitors of each MAPK decreased the IL-8 production stimulated by H_2O_2. These results indicate that H_2O_2 acts as an inducer of IL-8 production via activation of ERK, p38, and JNK. H_2O_2 deposited in periodontal tissue during inflammation against bacteria may accelerate tissue destruction via induction of IL-8 in PDL cells.

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