International Journal of Oral Biology, Vol. 34, No. 2, June 30 2009, p. 87~95

International Journal of Oral Biology

Production of pro-inflammatory cytokines by *Porphyromonas gingivalis* in THP-1 macrophagic cells

Eun-Kyoung Choi and In-Chol Kang*

Department of Oral Microbiology, Dental Science Research Institute, Chonnam National University Dental School, Gwangju 500-757, Korea

(received April 29, 2009 ; revised May 28, 2009 ; accepted June 5, 2009)

Porphyromonas gingivalis **is a major etiologic agent of chronic periodontitis and cytokines produced by macrophages play important roles in the pathogenesis of periodontal diseases. In this study we investigated the cytokine response of phorbol myristate acetate-differentiated THP-1 cells exposed to *P. gingivalis*. Compared with the prominent cell wall components of *P. gingivalis* (lipopolysaccharide and the major fimbrial protein FimA), live *P. gingivalis* stimulated much higher levels of cytokine production. In addition, whereas low multiplicity of infection challenges (MOI = 10) of *P. gingivalis* 381 stimulated high levels of monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and IL-1β, high dose challenges with this bacterium (MOI = 100) resulted in a substantially diminished production of MCP-1 and IL-6. Moreover, high MOI *P. gingivalis* challenges achieved only low levels of induction of MCP-1 and IL-6 mRNA. The decreased production of MCP-1 and IL-6 appeared to be mediated by *P. gingivalis* proteases, because high MOI challenges with congenic protease mutant strains of this microorganism (MT10 and MT10W) did not result in a diminished production of MCP-1 and IL-6. Similar to its protease mutant strains, leupeptin (a protease inhibitor)-treated *P. gingivalis* at high doses induced high levels of MCP-1 production. To examine the mechanisms underlying the diminished production of MCP-1 by *P. gingivalis* proteases, the activation of mitogen-activated protein (MAP) kinases and NF-κB was compared between the 381 and MT10W strains. Whilst high doses of both 381 and MT10W similarly activated the three members of the MAP kinase family, the DNA binding activity of NF-κB, as revealed by gel shift assays, was greatly increased only by MT10W. Taken together, our data indicate that *P. gingivalis* stimulates the production of high levels of TNF-α, IL-1β, IL-6, and MCP-1 but that high dose challenges with this bacterium result in a diminished production of MCP-1 and IL-6 via the protease-mediated suppression of NF-κB activation in THP-1 macrophagic cells.**

Key words : *Porphyromonas gingivalis*, THP-1 macrophagic cell, Periodontal disease, Inflammatory cytokine

Introduction

Porphyromonas gingivalis is a gram-negative, anaerobic, nonmotile rod. Of the suspected periodontal pathogens, *P. gingivalis* has been most consistently and strongly associated with chronic periodontitis (Socransky and Haffajee, 1992). This species markedly increased in prevalence and proportions in disease as compared to healthy sites and in sites demonstrating recent disease progression. Detection of *P. gingivalis* is indicative of increased risk for future disease progression, and successful clinical treatment of chronic periodontitis correlates with decreased levels (Ximenez-Fyvie et al., 2006). The host response to *P. gingivalis* is evident in increased serum antibody titers in subjects with periodontitis (Lamont and Jenkinson, 1998).

Periodontal diseases are infectious diseases and are associated with the subgingival growth of periodonto pathogens. However, much of the damage in the disease is

*Corresponding author: In-Chol Kang, Department of Oral Microbiology, Chonnam National University Dental School, 77 Yongbong-ro, Buk-gu, Gwangju 500-757, Korea, Tel.: +82-62-530-4851, Fax.: +82-62-530-4855, E-mail: ickang@jnu.ac.kr

actually the result of the host response to the bacteria, not the result of the bacteria themselves (Baker, 2000). Therefore, interactions of periodontopathogens with host cells and the resultant host cell responses are clearly of fundamental importance. *P. gingivalis* produces many cell components and macromolecules that have been proposed to function as virulence factors. These factors include lipopolysaccharide (LPS), various outer membrane proteins, fimbriae, and numerous end products of metabolism (Holt et al., 1999; Kim et al., 2008). Fimbriae play a critical role in mediating the bacterial interaction with host tissues, promoting bacterial adhesion to and invasion of the targeted sites (Nakagawa et al., 2005). Fimbriae and LPS of this bacterium have been implicated in both the initiation and progression of disease. Several studies have shown that *P. gingivalis* is able to induce the production of cytokines such as monocyte chemoattractant protein-1 (MCP-1), interleukin-1 (IL-1), IL-6, and tumor necrosis factor-α (TNF-α) from gingival fibroblasts, epithelial cells and macrophages (Hirose et al., 1996). These cytokines are thought to be important immunologic mediators in periodontal inflammation and the destruction of periodontal tissue. However, most studies have examined the response to bacterial components (e.g., LPS and fimbriae) rather than to live bacteria.

A dense infiltration of inflammatory cells, including monocytes/macrophages, occurs in the periodontal tissues of adult patients with periodontitis. Mononuclear phagocytes play an important role in the regulation of inflammatory host responses, in part through their ability to secrete mediators, particularly cytokines, in response to microorganisms and microbial products. Macrophages constitute a substantial proportion of the cells recovered from the gingival tissues, particularly the inflammatory tissues, of patients with periodontitis (Choi et al., 2006; Stoufi et al., 1987). These macrophages play an essential role in the development and progression of periodontal diseases.

The purpose of this study was to examine the production of various pro-inflammatory cytokines in THP-1 macrophagic cells in response to live *P. gingivalis* challenges under various conditions in order to gain more comprehensive insights on the cytokine responses elicited by *P. gingivalis*.

Materials and methods

Bacteria and growth conditions

The following *P. gingivalis* strains were grown in tryptic soy broth supplemented with yeast extract (1 mg/ml), cysteine (3.3 mM), hemin (5 μg/ml), dithiothreitol (0.1 mM), menadione (1 μg/ml): 381, a type strain; MT10, a *rgpA* mutant (Tokuda et al., 1998); MT10W, a *rgpA* and *kgp* mutant (Chen et al., 1999). All the strains were derived from 381. For the growth of all *P. gingivalis* strains, gentamicin (50 μg/ml) was added to the media. Erythromycin (10 μg/ml) was additionally added for *P. gingivalis* MT10, and MT10W. The bacteria were incubated anaerobically (85% N_2, 10% H_2 and 5% CO_2) at 37°C and bacteria in logarithmic growth phase were used in all experiments. For the preparation of heat killed *P. gingivalis*, bacteria were harvested, washed three times with phosphate-buffered saline (PBS), and resuspended in a small volume of PBS. Then the bacteria were exposed to heat (80°C for 30 min).

Cell culture

Human monocytic cells (THP-1) were purchased from American Type Culture Collection (USA). They were differentiated with 100 nM of phorbol myristate acetate (PMA) for 24 h in polystyrene culture plates at 37°C in a humidified atmosphere containing 5% CO_2. The culture medium consisted of RPMI 1640 medium (Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS) and 50 μg/ ml gentamicin.

LPS purification

P. gingivalis was grown under anaerobic conditions and harvested at the end of the logarithmic phase of growth. LPS extraction was achieved by the hot phenol-water method (Westphal and Jann, 1965). Briefly, the bacterial cell pellet was suspended in pyrogen-free water, and then an equal volume of 90% phenol at 60°C was added dropwise for 20 min and stirred constantly. The aqueous phase was separated by centrifugation at 7,000 × g for 15 min at 4°C and collected. This process was repeated, and the aqueous phase was pooled and dialyzed against deionized water for 3 days at 4°C. The dialyzed LPS preparation was then centrifuged at 200,000 × g for 1.5 h at 4°C in a Beckman ultracentrifuge (USA). The precipitate was suspended with 30 ml of pyrogen-free water, dialyzed against distilled water for 3 days, lyophilized, and stored at 4°C. The protein content of the purified LPS, determined by the method of Markwell et al. (1978), was less than 0.1%. LPS samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained for protein with Coomassie blue to confirm the purity of the LPS moieties.

Generation and purification of recombinant fimbrillin (rFimA)

P. gingivalis 381 genomic DNA was isolated and used as a template for amplifying the *fim*A gene by PCR. The primers used for PCR were constructed on the basis of the *fim*A gene sequence (Dickinson et al., 1988). The primer sequences with restriction sequences (underlined) are as follows: forward primers, 5'-CTCGAGATGGTATTGAAGACCAGC-3'; reverse primer, 5'-GGAATTCTTACCAAGTAGCATTCTGACC-3'. Plasmid pTrcHis A (Invitrogen) was used as the vector for the expression of FimA polypeptides. PCR and subsequent cloning of the *fim*A gene into the pTrcHis expression vector were carried out. Each amplified DNA was digested with *Xho*I and *EcoR*I (New England Biolabs,

USA), purified from an agarose gel by a gel extraction kit (Qiagen, Germany), and ligated into the pTrcHis A vector linearized with restriction enzyme *Xho*I and *Eco*RI. The ligated vector was transformed into *E. coli* TOP10 by electroporation, and the colonies were selected on LB agar plates containing 50 μg/ml ampicillin. After confirmation of the sequence, the plasmid was transformed into *E. coli* BL21. The cells harboring the plasmid were cultured to an A_{600} of 0.5 in LB medium containing 50 μg/ml ampicillin at 37°C. Then 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to induce protein expression, and the culture was incubated further at 30°C for 3 h. The *E. coli* cells were harvested by centrifugation and resuspended in 50 mM potassium phosphate buffer, pH 8.0, containing 0.1 M NaCl. Cell lysis was carried out by 10 cycles of 1 min of sonication and centrifuged at 20,000 × g for 30 min. The recombinant proteins were purified with the use of Pro-Bond™ resin column (Invitrogen), which contained Ni^{2+} as an affinity ligand. The purified protein gave a single band at approximately 42 kDa on SDS-PAGE (data not shown). Finally, the rFimA preparation was passed through a column containing agarose-immobilized polymyxin B (Detoxi-Gel, Pierce, USA). The rFimA preparation was tested for negativity of endotoxin contamination with the *Limulus* amebocyte lysate assay (BioWhittaker, USA).

Infection protocol

Bacteria were washed twice with PBS and once with RPMI medium. After resuspension in RPMI medium, the optical density of the bacterial suspension was measured at 600 nm, and further diluted to an optical density of 0.5 which corresponded to 5×10^8 CFU/ml. 1×10^6 PMA-differentiated THP-1 cells were infected with *P. gingivalis* in a final volume of 1 ml with various multiplicity of infections (MOI).

Measurement of cytokines from culture supernatants

The THP-1 culture supernatants were sampled and centrifuged at 100 × g for 5 min for clarification of debris. The levels of cytokines were quantified using commercial ELISA kits (R&D Systems, USA) according to the manufacturer's directions.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was prepared with Trizol reagent (Invitrogen) as specified by the manufacturer and was quantified spectctrophotometrically. First-strand cDNA was synthesized from 1 μg of RNA using random primers (Promega, USA) and Molony murine leukemia virus reverse transcriptase (Invitrogen). 2 μl of cDNA products were amplified in 25 μl volumes under a layer of mineral oil using a GeneAmp 2700 thermal cycler (Applied Biosystems, USA). Each PCR reaction mixture contained 50 mM KCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 1 U *Taq* DNA polymerase, and 0.5 μM of each primer. Each cycle consisted of denaturation at 94°C (30 s), annealing at 55°C (30 s), and extension at 72°C (60 s). The sequences of primers were 5'-CAGCCAGATGCAATCAATGC-3', 5'-GTGGTCCATGGAATCCTGAA-3' for MCP-1 (198 bp); 5'-TCAATGAGGAGACTTGCCTG-3', 5'-GATGAGTT GTCATGTCCTGC-3' for IL-6 (260 bp); 5'-CTTCTGCC TGCTGCACTTTGGA-3', 5'-TCCCAAAGTAGACCTG CCCAGA-3' for TNF-α (547 bp); 5'-CAGTGAAATGAT GGCTTATTAC-3', 5'-CTTTCAACACGCAGGACAGGT for IL-1β (548 bp); and 5'-AGCGGGAAATCGTGCGTG-3', 5'-CAGGGTACATGGTGGTGCC-3' for β-actin (300 bp). The PCR products of 10 μl were fractionated on 1.2% (w/v) agarose gels containing ethidium bromide, visualized by UV transillumination, and photographed.

Western blots

PMA-differentiated THP-1 cells in 3 cm dishes were lysed with 100 μl of Cell Lysis Buffer (Cell Signaling Technology, USA). 20 μg of each boiled sample was resolved by SDS-PAGE (10%) and transferred to a polyvinylidene difluoride membrane (Bio-Rad, USA). The membrane was probed with a 1:1000 dilution of rabbit anti-phospho-p38 polyclonal antibody and a 1:1500 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Cell Signaling Technology). Immunoreactive proteins were detected by enhanced chemiluminescence (LumiGLO, Cell Signaling Technology). The same membrane was successively stripped and reprobed with anti-phospho-c-Jun N-terminal kinase (JNK, 1:1000), anti-phospho-extracellular signal-regulated kinase (ERK, 1:1000), and anti-β-actin (Sigma, USA, 1:5000). The three anti-phospho antibodies were from Cell Signaling Technology.

Nuclear extract preparation and gel shift assays

For the preparation of nuclear extracts, cells were washed with ice-cold PBS and pelleted. The cell pellet was resuspended in hypotonic buffer (10 mM HEPES, pH 7.9 at 4°C, 0.5 mM KCl, 1.5 mM DTT, 0.2 mM PMSF) and incubated for 10 min on ice. Then, the cells were lysed by addition of 10% IGEPAL CA-630, followed by vigorous vortex for 10 s. Nuclei were pelleted and resuspended in low-salt buffer (20 mM HEPES, pH 7.9 at 4°C, 25% glycerol, 1.5 mM $MgCl_2$, 20 mM KCl, 0.2 mM EDTA, 0.5 mM DTT and 0.2 mM PMSF) and high-salt buffer (20 mM HEPES, pH 7.9 at 4°C, 1.5 mM $MgCl_2$, 0.8 M KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF) was added in a prop-wise fashion. After 30 min incubation at 4°C, the lysates were centrifuged, and supernatants containing the nuclear proteins were transferred to new vials. Protein concentrations of nuclear extracts were measured with DC protein assay kit (Bio-Rad). For a double-stranded NF-κB-binding DNA probe, two oligonucleotides were synthesizd (5'-CCGGTTAACAGAGGGGGCTTTCCGAG-3' and 5'-CCGGCTCGGAAAGCCCCCTCTGTTAA-3') (TaKaRa,

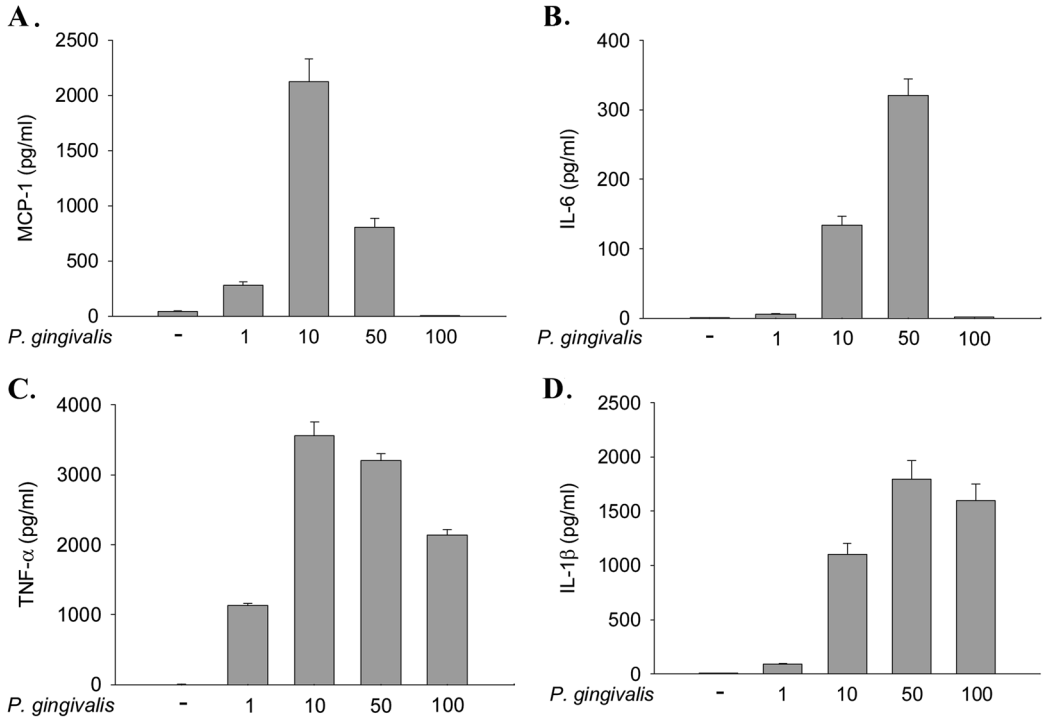

Fig. 1. Production of inflammatory cytokines by various doses of *P. gingivalis.* THP-1 cells were incubated with increasing doses (MOI = 1-100) of live *P. gingivalis* for 18 h. The culture supernatants were collected for cytokine measurement by ELISA. Data are expressed as the means ± S.D. of a representative experiment performed in triplicate.

Korea), annealed, and labeled with [α-^{32}P] dCTP and DNA polymerase I (Klenow fragment). The 5'-CCGG overhangs were for [α-^{32}P] dCTP incorporation. About 10 μg of nuclear extracts were incubated with 10,000 cpm of probe in 20 μl of reaction buffer containing 10 mM Tris-HCl (pH 7.6), 50 mM KCl, 1 mM EDTA, 5% glycerol, 1 mM DTT and 200 ng of poly(dI·dC) for 30 min at room temperature. Specific binding of NF-κB was controlled by competition with a 50-fold excess of cold NF-κB probe or irrelevant cAMP response element-binding protein (CREB) probe (Promega). The DNA-protein complexes were separated on 4% polyacrylamide gels. The gels were dried and subjected to autoradiography.

Results

Production of inflammatory cytokines by various doses of *P. gingivalis*

The PMA-differentiated THP-1 cells were challenged with increasing doses of *P. gingivalis* 381 for 18 h, and the concentrations of MCP-1, TNF-α, IL-6, and IL-1β in the collected supernatants were measured by ELISA. At a multiplicity of infection (MOI) of 1:1, little cytokine production was stimulated except for TNF-α. Strong responses of cytokine production were observed with MOIs of 10-50. However, high dose challenges (MOI=100) resulted in profoundly diminished production of MCP-1 and IL-6 (Fig. 1).

mRNA expression of inflammatory cytokines by various doses of *P. gingivalis*

In order to examine the induction of MCP-1, IL-6, TNF-α, and IL-1β at the steady-state mRNA level, RT-PCR analysis was performed on RNA extracted from the THP-1 cells that had been stimulated by increasing doses of *P. gingivalis* 381 for 2 h and 6 h. The mRNA levels of TNF-α and IL-1β were readily increased by the range of doses (MOI=10-100) throughout the time course. Although the mRNA expression levels of MCP-1 and IL-6 were also increased by *P. gingivalis*, the mRNA levels with high dose challenges were considerably lower than those with low dose challenges (Fig. 2).

Production of MCP-1 and TNF-α by *P. gingivalis*, LPS and rFimA

As LPS and fimbriae of *P. gingivalis* are well-known cytokine inducers, the cytokine production profile was investigated in THP-1 cells exposed to live *P. gingivalis*, its LPS, or its rFimA. THP-1 cells were challenged with increasing doses of live *P. gingivalis*, LPS, or rFimA for 18 h, and the concentrations of MCP-1 and TNF-α in the collected supernatants were measured by ELISA. The mRNA expression was also assessed by RT-PCR. Although both LPS and rFimA induced the production of MCP-1 and TNF-α, the amounts of the cytokines were much smaller compared to *P. gingivalis*-stimulated cytokine production

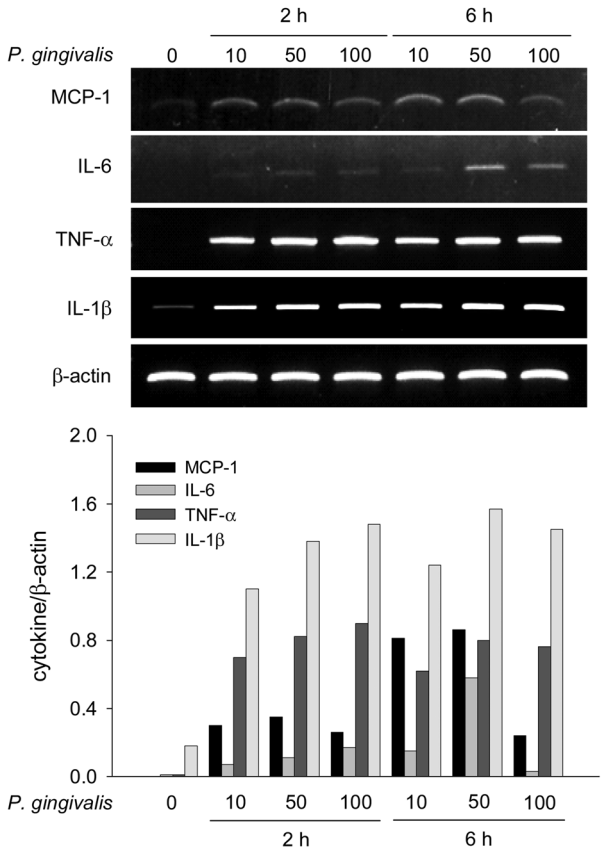

Fig. 2. mRNA expression of inflammatory cytokines by various doses of *P. gingivalis*. THP-1 cells were incubated with various doses (MOI = 10-100) of live *P. gingivalis* 381 for 2 h or 6 h. Total RNA was isolated and levels of MCP-1, IL-6, TNF-α, and IL-1β mRNA expression were determined by RT-PCR (a). Results are expressed as cytokine/β-actin ratio by densitometric analysis (b).

(Fig. 3a). The results of the mRNA expression were generally consistent with those of the protein production. At 18 h postinfection, live *P. gingivalis* strongly stimulated the mRNA induction of MCP-1 and TNF-α in THP-1 cells. The mRNA levels were considerably lower in LPS or FimA-stimulated THP-1 cells. Especially, the mRNA expression of MCP-1 by rFimA was minimal with all dose range (Fig. 3b).

Comparison of cytokine-inducting activity between live and killed *P. gingivalis*

To evaluate the effect of bacterial viability on the cytokine production by *P. gingivalis*, THP-1 cells were incubated with the same number of live or heat-killed *P. gingivalis* 381 for 24 h and 48 h. Not only live but also killed *P. gingivalis* strongly increased the production of MCP-1 and TNF-α. It was notable that killed bacteria resulted in a robust MCP-1 production at high dose challenges, where live bacteria achieved no MCP-1 production (Fig. 4).

Production of inflammatory cytokines by protease mutant strains of *P. gingivalis*

To gain more insight into the mechanism by which *P. gingivalis* stimulates THP-1 cells to produce the inflammatory cytokines, the cytokine production by several mutant strains was compared. THP-1 cells were challenged with 381 (wild type), MT10 (*rgpA* mutant), or MT10W (*rgpA* and *kgp* mutant) for 18 h, and the concentrations of MCP-1, IL-6, TNF-α, and IL-1β in the supernatants were measured by ELISA. As shown in Fig. 5, compared to wild type strain 381, the protease mutants (MT10 and MT10W) resulted in increased production of MCP-1 and IL-6 at high dose challenges (MOI=100).

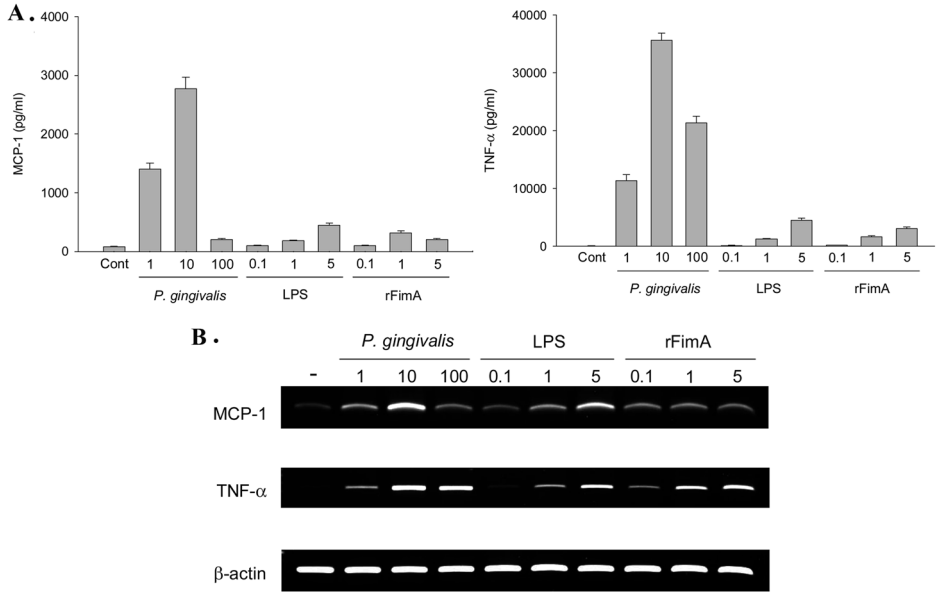

Fig. 3. Production of MCP-1 and TNF-α by *P. gingivalis* or cell wall components (LPS and rFimA). THP-1 cells were incubated with *P. gingivalis* (MOI=1-100), *P. gingivalis* LPS, or rFimA (0.1-5 μg/ml) for 18 h. (a) MCP-1 and TNF-α concentrations of the culture supernatants were measured by ELISA. Data are expressed as the means ± S.D. of a representative experiment performed in triplicate. (b) Total RNA was isolated and levels of MCP-1 and TNF-α mRNA expression were determined by RT-PCR.

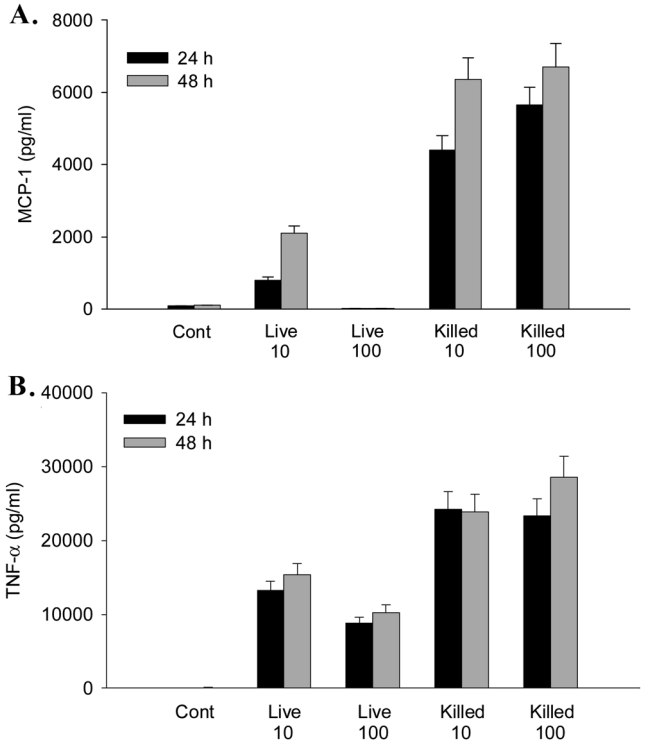

Fig. 4. Comparison of cytokine-inducing activity between live and killed *P. gingivalis.* THP-1 cells were incubated with live or heat-killed *P. gingivalis* for 24 h and 48 h. The supernatants were collected and concentrations of MCP-1 and TNF-α were determined by ELISA. Data are expressed as the means ± S.D. of a representative experiment performed in triplicate.

MCP-1 production by leupeptin-treated *P. gingivalis*

Leupeptin is a protease inhibitor especially effective for gingipain R. It was of interest to examine the MCP-1 production by leupeptin-treated *P. gingivalis*. Leupeptin-treated *P. gingivalis* was prepared by incubating *P. gingivalis* in the presence of leupeptin (1 mM) for 20 h. Interestingly, leupeptin-treated *P. gingivalis*, induced high levels of MCP-1 production at high doses (Fig. 6a). The similar results were also seen at the steady-state mRNA levels, in which leupeptin-treated *P. gingivalis*, did not decrease the MCP-1 mRNA expression (Fig. 6b).

Activation of MAP kinases by *P. gingivalis* 381 and MT10W

In order to explore the underlying mechanisms responsible for the diminished MCP-1 production by *P. gingivalis* 381, the activation of MAP kinases was compared between 381 and MT10W. THP-1 cells were incubated with *P. gingivalis* 381 or MT10W (MOI=100) for up to 2 h. The results showed that high doses of both 381 and MT10W stimulated the activation of three members of MAP kinase family to the similar extent (Fig. 7).

DNA binding activity of NF-κB by *P. gingivalis* 381 and MT10W

The MCP-1 and IL-6 genes are transcriptionally regulated via NF-κB binding to specific DNA sequences within their promoters. Therefore, the DNA binding activity of NF-κB was compared between 381 and MT10W by gel shift assays.

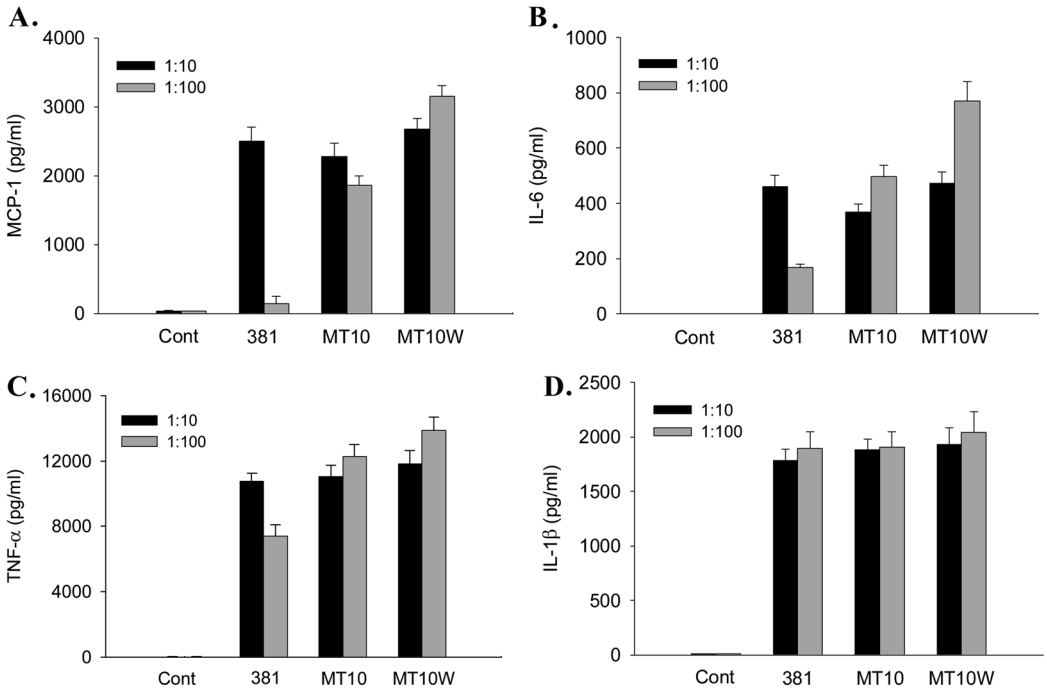

Fig. 5. Production of inflammatory cytokines by protease mutant strains of *P. gingivalis.* THP-1 cells were incubated with 381, MT10, or MT10W strains of *P. gingivalis* at MOIs of 10 or 100 for 18 h. The supernatants were collected for cytokine analysis by ELISA. Data are expressed as the means ± S.D. of a representative experiment performed in triplicate.

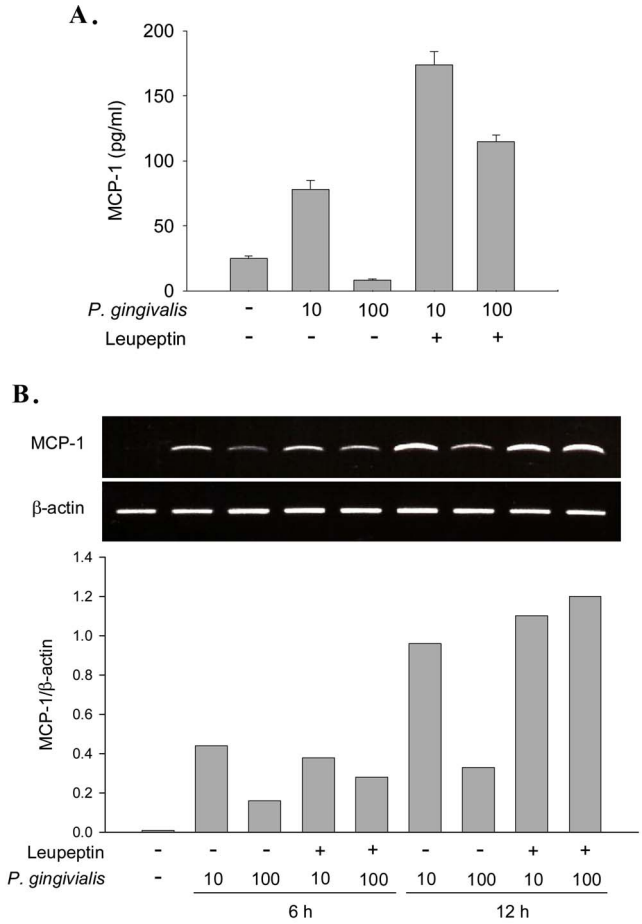

Fig. 6. MCP-1 production by leupeptin-treated *P. gingivalis*. (a) THP-1 cells were infected with leupeptin-treated *P. gingivalis*. After 8 h, MCP-1 concentrations of culture supernatants were measured by ELISA. Data are expressed as the means ± S.D. of a representative experiment performed in triplicate. (b) Total RNA was isolated from the (a) experiments, and levels of MCP-1 mRNA were determined by RT-PCR.

Inducible NF-κB DNA binding activities were readily detected after stimulation with MT10W. However, *P. gingivalis* 381 barely induced NF-κB DNA binding activity (Fig. 8).

Discussion

Bacterial infections typically result in the production of specific pro-inflammatory cytokines and chemokines, leading to host defense of the invading microbes. Macrophages constitute a substantial proportion of the cells recovered from the gingival tissues, particularly the inflammatory tissues, of patients with periodontitis (Stoufi et al., 1987). Although these macrophages play protective roles in the elimination of periodontopathogens from gingival tissues, they may contribute to periodontal pathogensis by releasing many inflammatory cytokines, including MCP-1, TNF-α, IL-1β, and IL-6. In the present

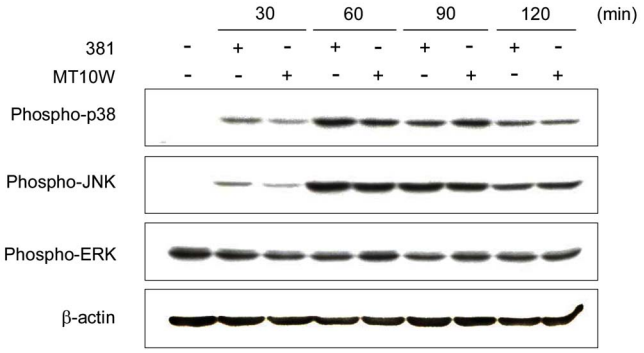

Fig. 7. Activation of MAP kinases by *P. gingivalis* 381 and MT10W. THP-1 cells were infected with *P. gingivalis* 381 or MT10W (MOI = 100) for the indicated time periods. Cell lysates were prepared and Western blot analysis was performed for phospho-p38, phospho-JNK, or phospho-ERK.

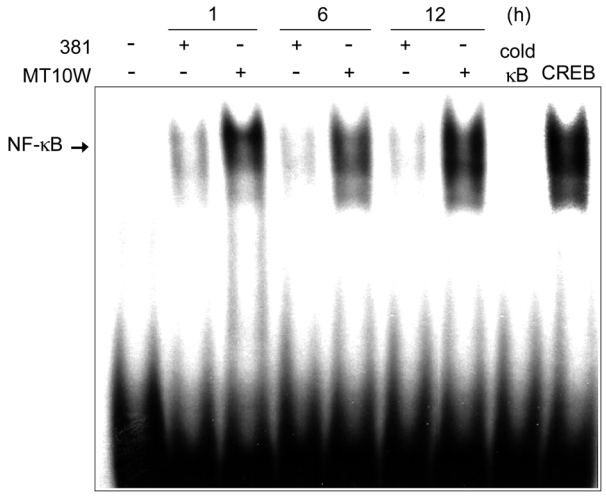

Fig. 8. DNA binding activity of NF-κB by *P. gingivalis* 381 and MT10W. THP-1 cells were infected with *P. gingivalis* 381 and MT10W (MOI = 100) for the indicated time periods. Nuclear extracts were prepared and the DNA binding activity of NF-κB was determined by gel shift assays. NF-κB specificity was controlled with a 50-fold excess of cold NF-κB probe or irrelevant CREB probe.

study, *P. gingivalis* strongly induced the production of all the cytokines measured, TNF-α, IL-1, IL-6, and MCP-1, in THP-1 macrophagic cells. TNF-α and IL-1β are the most important pro-inflammatory cytokines and they are potential markers of the progression and severity of periodontitis. IL-6 plays an important role in many chronic inflammatory diseases, being involved in leukocyte recruitment, apoptosis, and T-cell activation (Hou et al., 1995; Assuma et al., 1998). MCP-1 increases in the crevicular fluids of adult periodontal patients with severity of the disease (Hanazawa et al., 1993).

LPS and fimbriae of *P. gingivalis* have been known to stimulate host cells to induce various pro-inflammatory cytokines (Hajishengallis et al., 2006). In this study, although both LPS and FimA induced the production of

MCP-1 and TNF-α, the amounts of the cytokines were much smaller compared to *P. gingivalis*-stimulated cytokine production. Moreover, the amount of LPS and FimA present in live *P. gingivalis* cultures used in the *in vitro* and *in vivo* assays is much lower than the amount used with purified LPS or FimA. It is estimated that 1×10^8 *P. gingivalis* corresponds to 0.36 μg of LPS and 0.15 μg of FimA at most. These suggest that the strong cytokine production in response to live *P. gingivalis* is not solely due to its LPS and FimA (Zhou et al., 2005).

It was notable that high dose challenges of live *P. gingivalis* (MOI=100) resulted in substantially diminished production MCP-1 and IL-6. Moreover, the mRNA levels with high dose challenges were considerably lower than those with low dose challenges, indicating that the diminished production of MCP-1 and IL-6 is not due to degradation of secreted cytokines by the proteolytic activity of high dose *P. gingivalis.*

As protease mutants strains of *P. gingivalis* did not result in diminished production of MCP-1 at high dose challenges, involvement of proteases in the suppression of MCP-1 production was determined. Like protease mutants, leupeptin-treated *P. gingivalis* and heat-killed *P. gingivalis* did not result in attenuated production of MCP-1 at high dose challenges. These results suggest that the proteases are responsible for the attenuated production of MCP-1 at high dose challenges. As a matter of fact, the high protease activity of *P. gingivalis* has been singled out as an important virulence trait of this organism (Sugawara et al., 2000). The activation of MAP kinases and NF-κB plays important roles in the production of most inflammatory cytokines including MCP-1 (Chun and Surh, 2004; Elewaut et al., 1999). Therefore, the activation of MAP kinases and NF-κB was compared between 381 and MT10W. There was no difference in activation of MAP kinases. In contrast, NF-κB DNA binding activities were readily detected only with MT10W, not with 381. These results suggest that high doses of *P. gingivalis* inhibit MCP-1 production via bacterial proteases-mediated suppression of NF-κB activation in THP-1 macrophagic cells. This is the first report indicating that bacteria can inhibit production of certain cytokines in a bacterial proteases-dependent manner. Further study is needed to elucidate the detailed mechanisms of the NF-κB suppression.

Acknowledgements

This study was financially supported by Korea Research Foundation (KRF-2006-312-E00138).

References

Assuma R, Oates T, Cochran D, Amar S, Graves DT. IL-1 and TNF antagonists inhibit the inflammatory response and bone loss in experimental periodontitis. J Immunol. 1998;160:403-9.

Baker PJ. The role of immune responses in bone loss during periodontal disease. Microbes Infect. 1998;2:1181-92.

Chen W, Kuramitsu HK. Molecular mechanism for the spontaneous generation of pigmentless *Porphyromonas gingivalis* mutants. Infect Immun. 1999;67:4926-30.

Choi EK, Oh BH, Kang IC. Cyclooxygenase-2 induction in *Porphyromonas gingivalis* -infected THP-1 monocytic cells. Int J Oral Biol. 2006;31;21-6.

Chun KS, Surh YJ. Signal transduction pathways regulating cyclooxygenase-2 expression: potential molecular targets for chemoprevention. Biochem Pharmacol. 2004;68:1089-100.

Dickinson DP, Kubiniec MK, Yoshimura F, Genco RJ. Molecular cloning and sequencing of the gene encoding the fimbrial subunit protein of *Bacteroides gingivalis*. J Bacteriol. 1988;170:1658-65.

Elewaut D, DiDonato JA, Kim JM, Truong F, Echmann L, Kagnoff MF. NF-κB is a central regulator of the intestinal epithelial cell innate immune response induced by infection with enteroinvasive bacteria. J Immunol. 1999;163:1457-66.

Hanazawa S, Kawata Y, Takeshita A, Kumada H, Okithu M, Tanaka S, Yamamoto Y, Masuda T, Umemoto T, Kitano S. Expression of monocyte chemoattractant protein 1 (MCP-1) in adult periodontal disease: Increased monocyte chemotactic activity in crevicular fluids and induction of MCP-1 expression in gingival tissues. Infect Immun. 1993;61:5219-24.

Hajishengallis G, Tapping RI, Harokopakis E, Nishiyama S, Ratti P, Schifferle RE, Lyle EA, Triantafilous M, Triantafilou K, Yoshimura F. Differential interactions of fimbriae and lipopolysaccharide from *Porphyromonas gingivalis* with the Toll-like receptor 2-centred pattern recognition apparatus. Cell Microbiol. 2006;10:1557-70.

Hirose K, Isogai E, Mizugai H, Miura H, Ueda I. Inductive effect of *Porphyromonas gingivalis* fimbriae on differentiation of human monocytic tumor cell line U937. Oral Microbiol Immunol. 1996;11:62-4.

Holt SC, Kesavalu L, Walker S, Genco CA. Virulence factors of *Porphyromonas gingivalis*. Periodontol 2000. 1999;20:168-238.

Hou LT, Liu CM, Rossomando EF. Crevicular interleukin-1 beta in moderate and severe periodontitis patients and the effect of phase I periodontal treatment. J Clin Periodontol. 1995;22:162-7.

Kim SR, Park HJ, Bae SK, Park JH, Kim HS, Koo TH, Bae MK. *Porphyromonas gingivalis* lipopolysaccharide increases monocyte adhesion to microvascular endothelium by induction of adhesion molecules. Int J Oral Biol. 2008;33:149-54.

Lamont RJ, Jenkinson HF. Life below the gum line: pathogenic mechanisms of *Porphyromonas gingivalis*. Microbiol Mol Biol Rev. 1998;62:1244-63.

Markwell MA, Haas SM, Bieber LL, Tolbert NE. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal Biochem. 1978;87:206-10.

Nakagawa I, Amano A, Inaba H, Kawai S, Hamada S. Inhibitory effects of *Porphyromonas gingivalis* fimbriae on interactions between extracellular matrix proteins and

cellular integrins. Microbes Infect. 2005;7:157-63.
Socransky SS, Haffajee AD. The bacterial etiology of destructive periodontal disease: current concepts. J Periodontol. 1992;63: 322-31.
Stoufi ED, Taubman MA, Ebersole JL, Smith DJ, Stashenko PP. Phenotypic analyses of mononuclear cells recovered from healthy and diseased human periodontal tissues. J Clin Immunol. 1987;7:235-45.
Sugawara S, Nemoto E, Tada H, Miyake K, Imamura T, Takada H. Proteolysis of human monocyte CD14 by cysteine proteases (gingipains) from *Porphyromonas gingivalis* leading to lipopolysaccharide hyporesponsiveness. J Immunol. 2000;165: 411-8.
Tokuda M, Karunakaran T, Duncan M, Hamada N, Kuramitsu H. Role of Arg-gingipain A in virulence of *Porphyromonas gingivalis*. Infect Immun. 1998;66:1159-66.
Westphal O, Jann K. Bacterial lipopolysaccharides: extraction with phenol water and further applications of the procedure. Methods Carbohydr Chem. 1965;5:83-91.
Ximenez-Fyvie LA, Almaguer-Flores A, Jacobo-Soto V, Lara-Cordoba M, Sanchez-Vargas LO, Alcantara-Maruri E. Description of the subgingival microbiota of periodontally untreated Mexican subjects: chronic periodontitis and periodontal health. J Periodontol. 2006;**77**:460-71.
Zhou Q, Desta T, Fenton M, Graves DT, Amar S. Cytokine profiling of macrophages exposed to *Porphyromonas gingivalis*, its lipopolysaccharide, or its FimA protein. Infect Immun. 2005;73:935-43.