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Plasminogen Activator Inhibitor Type 1 Expression Induced by Lipopolysaccharide of *Porphyromonas gingivalis* in Human Gingival Fibroblast

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In the gingival tissues of patients with periodontitis, inflammatory responses are mediated by a wide variety of genes. In our previous screening study, plasminogen activator inhibitor type 1 (PAI-1) mRNA binding protein expression was increased in gingiva from periodontitis patients. In this study, we further investigated the signaling pathway involved in PAI-1 expression induced by *Porphyromonas gingivalis* LPS (Pg LPS) in human gingival fibroblasts (HGF). When HGFs were treated with Pg LPS, both PAI-1 mRNA expression and PAI-1 protein were induced in a dose-dependent manner. Pg LPS induced NF-κB activation and the expressions of PAI-1 mRNA and protein were suppressed by pretreating with a NF-κB inhibitor. Pg LPS also induced ERK, p38, and JNK activation, and Pg LPS-induced PAI-1 expression was inhibited by ERK/p38/JNK inhibitor pretreatment. In conclusion, Pg LPS induced PAI-1 expression through NF-κB and MAP kinases activation in HGF.

Keywords: *Porphyromonas gingivalis*, PAI-1, periodontitis

Introduction

Periodontal disease consists of a group of infections leading to the inflammation of gingival tissues and destruction of periodontal tissues (Darveau, 2010). Mechanisms of host response in the periodontal tissues are complex and involve many interactions. The interaction of bacterial antigen with the host immune system is believed to be the basis for the destructive inflammatory response found in periodontal disease. We recently reported that the gene expression of plasminogen activator inhibitor type 1 (PAI-1) mRNA-binding protein was greatly increased in inflammatory gingiva using annealing control primer-polymerase chain reaction (ACP-PCR) (Na *et al.*, 2012). This led us to hypothesize that PAI-I is important in periodontitis, so we studied the signaling mechanism involved in PAI-1 expression induced by Pg LPS in human gingival fibroblast (HGF).

PAI-1 is related to the inflammatory response by regulating cell migration (McMahon *et al.*, 2001; Czekay *et al.*, 2003). Research about the relationship between plasminogen activator (PA) system and periodontal disease has focused on PAI-2 among the PA system inhibitors. PAI-2 expression was changed in inflamed periodontal tissue compared to normal periodontal tissue (Kinnby, 2002; Guan *et al.*, 2011). However, a few studies have reported PAI-1 in inflammatory gingiva (Xiao *et al.*, 1998; Kinnby *et al.*, 1999; Deppe *et al.*, 2010), and research about the signaling pathway for PAI-1 expression is sparse.

Among the various bacterial species associated with the development of periodontitis, *Porphyromonas gingivalis* is suspected to be an important cause of the chronic form of periodontitis (Yu *et al.*, 2011). *P. gingivalis* produces a broad spectrum of virulence factors, including outer membrane vesicles, adhesins, lipopolysaccharides (LPS), hemolysins and proteinases (Curtis *et al.*, 2001; Imamura, 2003; Furuta *et al.*, 2009; Kocgozlu *et al.*, 2009; Darveau, 2010). In particular, LPS is believed to contribute to host colonization, evasion of immune defense mechanisms and destruction of periodontal tissues (Bainbridge and Darveau, 2001; Ogawa *et al.*, 2007; Slaney and Curtis, 2008).

In this study, we hypothesized that Pg LPS induces the expression of PAI-1, possibly associated with chronic periodontitis. Thus, we investigated the expression of PAI-1 induced by Pg LPS in human gingival fibroblasts and further described its signaling pathways on the production of PAI-1.

Materials and Methods

Bacterial culture

P. gingivalis A7A1-28 (ATCC 53977) was cultured in a brain heart infusion broth containing 5 mg/ml of hemin and 0.5 mg/ml of vitamin K at 37°C in an anaerobic chamber in an atmosphere containing 90% N_2, 5% H_2, and 5% CO_2.

LPS purification

P. gingivalis was grown under anaerobic conditions to an OD 650 of 1.5 and harvested at the end of the logarithmic phase of growth. LPS extraction was achieved by the hot-phenol-water method (Kristoffersen *et al.*, 1971). Bacterial cell pellet was suspended in pyrogen-free water, and then equal volume of 90% phenol at 60°C was added drop wise for 20 min and stirred constantly. The aqueous phase was

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separated by centrifugation at 7,000 rpm 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 40,000 rpm for 1.5 h at 4°C in a Beckman Ultracentrifuge (Beckman Coulter, 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. LPS samples were separated by SDS-PAGE and stained for protein with Coomassie blue to confirm the purity of the LPS moieties.

Cell culture

Human gingival fibroblasts (HGF, ATCC CRL2014) were purchased from American Type Culture Collection (USA). They were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (FBS) (Life Technologies, Scotland), 100 U/ml penicillin and 100 μg/ml of streptomycin, and were incubated at 37°C in a humidified atmosphere of 5% CO_2. For mRNA expression analysis, HGF cells were grown in 6-well plates. The cells were treated for dose-dependent and time-dependent with Pg LPS. For PAI-1 protein analysis, HGF cells were seeded into 24-well tissue culture plates overnight. Cells were treated with 2 μg of Pg LPS from 4 h to 24 h and supernatants were collected stored at -20°C until analysis.

Quantitative real-time PCR

HGF cells (5×10^3 cells/well) were grown in 6-well plates each time. Total RNA was prepared by using TRIzol (Invitrogen, USA) according to the manufacturer's instructions. The reverse transcription (RT) of total RNA to cDNA was performed using AccuPower RT PreMix (Bioneer Co., Korea). The mRNA expression levels were quantified by real-time PCR using a Light Cycler instrument (Roche Applied Science, Germany) with the SYBR Green PCR Master Mix (Qiagen, USA) according to the manufacturer's instructions. The primer sequences for RT-PCR primer for PAI-1 were: forward (5′-CCA GCA GCA GAT TCA AGC AG-3′), and reverse (5′-GGC AGT TCC AGG ATG TCG TAG-3′) and the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were: forward (5′-AGT ATT CGG GGA AAC CT-3′), and reverse (5′-AAG CAG AGT CTC GGG A-3′). The mRNA levels of target genes were normalized to the relative amounts of GAPDH.

Measurement of PAI-1

The amounts of PAI-1 released to the culture media after Pg LPS stimulation were analyzed by using an ELISA kit from R&D systems (USA). A standard or sample solution was added to an ELISA well plate. After incubation for 2 h at room temperature, polyclonal anti-PAI-1 antibody conjugated with horseradish peroxidase was added to the solution and incubated for 2 h at room temperature. A substrate solution containing hydrogen peroxidase and chromogen was added and allowed to react for 30 min. The levels of cytokine expression were assessed by ELISA reader (TECAN Inc., Austria) at 450 nm. Each densitometric value expressed as mean±standard deviation (SD) was obtained from three independent experiments.

NF-κB activation assay

The nuclear extracts were prepared from HGF after Pg LPS stimulation for 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, and 12 h. NF-κB activation assay was carried out using an NF-κB p50/p65 Transcription Factor Assay (Cayman, USA). Cells were washed with ice-cold phosphate buffered saline (PBS), and the cell pellets were suspended in hypotonic lysis buffer (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5–5 mM DTT, 0.1% Triton X-100, Protease inhibitor cocktail) and incubated for 15 min on ice to isolate nuclear pellets. The nuclear pellets were suspended in extract buffer [20 mM HEPES; pH 7.9, 1.5 mM $MgCl_2$, 0.42 M NaCl, 0.2 mM EDTA, 0.5–5 mM DTT, 1.0% Igepal CA-630, 25% (v/v) glycerol, Protease inhibitor] and incubated for 30 min on ice. The lysates were centrifuged and the supernatants containing the nuclear proteins were used for the essay. The nuclear samples, negative control sample, positive control sample, and competitive oligonucleotide control sample were added to each plate well coated with the capture probe, a double stranded biotinylated oligonucleotide containing the flanked DNA binding consensus sequence for NF-κB (5′-GGGAC TTTCC-3′) for 2 h. The active NF-κB protein immobilized on the capture probe bound to the streptavidin plate well was detected with specific primary antibody, a rabbit anti-NF-κB p65. A horseradish peroxidase-conjugated anti-rabbit antibody was added to each well as secondary antibody and incubated for 30 min at room temperature. The pre-equilibrated 3,3′,5,5′-tetramethybenzidine (TMB) substrate solution was added to each well. The plate was incubated at room temperature for 10 min. The absorbance of each well was measured at 450 nm with a microplate reader (TECAN, Australia). Each value expressed as mean±SD was obtained from three independent experiments.

Separation of nuclear and cytosolic fractions

Nuclear and cytosolic fractions were separated with a commercially available kit according to the manufacturer's protocol (Abnova, Taiwan). Cells were scraped off the dish and centrifuged at 300×g for 5 min at 4°C. The resulting pellet was resuspended in ice-cold PBS/Phosphatase Inhibitor Solution, and centrifuged at 300×g for 5 min at 4°C. The pellet was resolved in ice-cold 1X hypotonic buffer and incubated for 15 min at 4°C. After addition of 10% Nonidet P-40 Assay Reagent, the pellet was gently mixed by pipetting and then centrifuged for 30 sec at 4°C. The supernatant containing the cytosolic fraction was transferred to a new tube. The resulting pellet was suspended in ice-cold Complete Nuclear Extraction Buffer (1X) (with protease and phosphatase inhibitors) and incubated for 15 min at 4°C. After centrifugation for 10 min at 14,000×g at 4°C, the resulting supernatant was obtained as the nuclear fraction. The purity of the nuclear and cytosolic fraction was ensured by immunoblotting with Lamin B and β-actin.

Western blot analysis

Immunoblot analysis was performed with antibodies specific for phospho-p65, MAPK, and phosphor-MAPK (p44/42-

Thr202/Tyr204, JNK-Thr183/Tyr185, and p38-Thr180/Tyr182) (Cell Signaling, USA); p65, Lamin B, and β-actin. (Santa Cruz, USA); (Cell Signaling). Antibodies were detected by ECL enhanced chemiluminescence system.

In vitro immunocytochemistry

Cells were fixed in 4% paraformaldehyde for overnight. Fixed cells were washed three times in PBS for 10 min. Cells were permeabilized with wash buffer (0.2% Triton-X in PBS) for 5 min and incubated for 1 h in wash buffer containing 1.5% serum. The primary antibody for PAI-1 was incubated for 2 h in 1.5% normal blocking serum in PBS at room temperature. Cells were washed thoroughly and then incubated with biotin-conjugated secondary antibody for 2 h. Cells were washed with wash buffer and combined with avidin-biotin enzyme reagents for 1 h. After washing, cells were incubated in peroxidase substrate containing diaminobenzidine until the reaction product was optimized. Cells were washed in deionized water to stop the reaction. After counter staining with hematoxylin, cells were dehydrated through alcohols and xylenes, mounted onto slide glass in malinol, and observed by light microscopy.

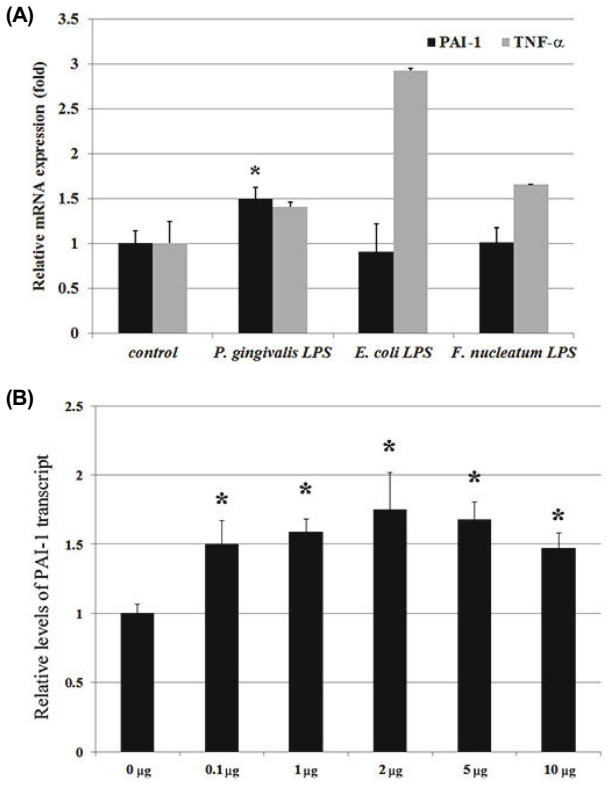

Fig. 1. (A) PAI-1 mRNA expression after stimulation with various bacterial LPS. HGF was treated with *E. coli*, *F. nucleatum* or *P. gingivalis* for 2 h and total RNA was prepared for PAI-1 and TNF-α mRNA expression analysis. (B) PAI-1 mRNA expression after stimulation with various concentrations of Pg LPS. HGF was treated with each concentration of Pg LPS for 2 h, and total RNA was prepared for PAI-1 mRNA expression analysis. Real time-PCR was performed as described in 'Materials and Methods'. The asterisk indicates a significant difference from the control (P<0.05).

Reagents

Pyrrolidine dithiocarbamate (PDTC), an NF-κB inhibitor, mitogen-activated protein (MAP) kinase inhibitors including PD98059 for extracellular signal-regulated kinase (ERK), SB203580 for p38 MAP kinase (p38), and SP600125 for c-Jun N-terminal protein kinase (JNK) were purchased from Calbiochem (USA).

Statistical analysis

Data were analyzed using Prism GraphPad v5 (GraphPad Software, Inc, USA). The Mann-Whitney U test was used to compare nonparametric unpaired data. Null hypotheses of no difference were rejected if P values were less than .05.

Results

PAI-1 mRNA expression by Pg LPS concentrations

To investigate the specificity of the Pg LPS for PAI-1 induction in association with chronic periodontitis, we first examined whether the LPSs derived from other pathogens induce PAI-1 expression in HGF. As shown in Fig. 1A, Pg LPS specifically induced PAI-1 expression compared to *E. coli* and *F. nucleatum* LPS. TNFα expression is shown as a positive control for LPS responsiveness.

To optimize the infection condition, HGF were stimulated with 0.1, 1, 2, 5, or 10 μg of Pg LPS for 2 h. PAI-1 mRNA expression was induced by all concentration of Pg LPS stimulations and proportionally increased when Pg LPS concentration was increased up to 2 μg. For further experiments, 2 μg of Pg LPS stimulation was selected (Fig. 1B).

Pg LPS induces mRNA expression and protein synthesis of PAI-1 in HGF

To clarify the time kinetics of PAI-1 mRNA expression, HGF were treated with Pg LPS for 30 min, 1 h, 2 h, 4 h, 8 h, 18 h, and 24 h. After total RNA preparation, PAI-1 mRNA expression was examined by real time-PCR. The PAI-1 mRNA increased 1 h after Pg LPS treatment, reached its maximum at 8 h, and declined thereafter (Fig. 2A).

To determine the kinetics of PAI-1 protein production, HGF were challenged with Pg LPS, and the culture supernatants were collected at the indicated time points and analyzed for PAI-1 protein production by ELISA. PAI-1 production gradually increased from 4 h to 24 h after Pg LPS treatment (Fig. 2B). To confirm the PAI-1 protein production by Pg LPS, HGF were treated with Pg LPS for 8 h, 12 h, and 24 h and were stained with PAI-1. As shown in Fig. 2C, PAI-1 protein expression greatly increased after 8 h and subsequently increased thereafter until 24 h. These data indicate that Pg LPS can induce mRNA expression and protein production of PAI-1 in HGF.

Pg LPS-induced PAI-1 expression is mediated by the activation of NF-κB in HGF

To clarify a possible involvement of NF-κB in Pg LPS-induced PAI-1 gene expression, the activation of NF-κB in Pg LPS-treated HGF was measured at various time intervals.

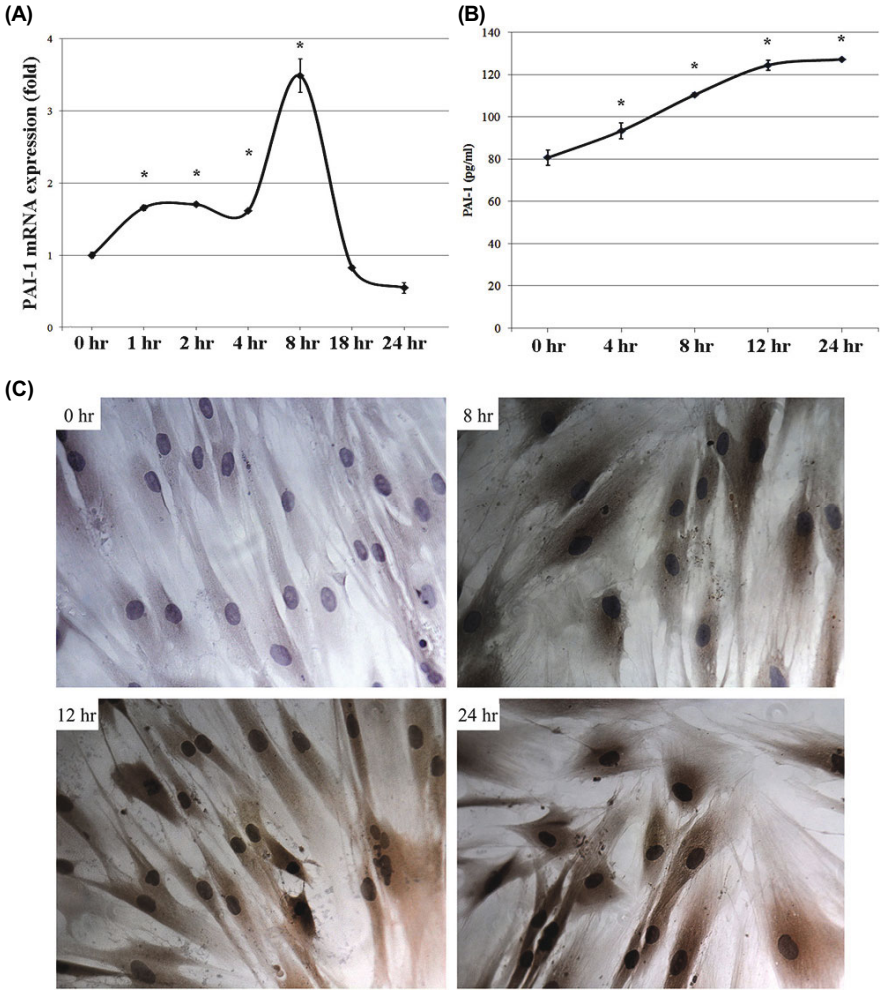

Fig. 2. Time kinetics of Pg LPS-induced PAI-1 mRNA expression. (A) HGF were treated with Pg LPS, and total RNA was prepared at the times indicated. Real time-PCR was performed as described in 'Materials and Methods'. Time kinetics of Pg LPS-induced PAI-1 protein production. (B) HGF were treated with Pg LPS, and the culture supernatants were collected at the times indicated. The levels of PAI-1 production in culture supernatants were measured by ELISA. Values are expressed as Mean±the SD obtained from three independent experiments. Detection of PAI-1 by immunocytochemistry in HGF (C). HGF were treated with Pg LPS for indicated time and immunostained. The asterisk indicates a significant difference from the control (P<0.05).

Pg LPS markedly enhanced the binding of nuclear protein to the consensus sequences of the binding site for NF-κB (Fig. 3A). Activation of the NF-κB associated with nuclear translocation of the p65 was further confirmed by western blot analysis. NFκB was translocated from the cytoplasm to the nucleus after 30 min of LPS stimulation (Fig. 3B).

To determine whether NF-κB is critical for PAI-1 gene expression by Pg LPS, HGF were pretreated with PDTC (50 μM), a NF-κB inhibitor, and then stimulated with Pg LPS. PAI-1 mRNA expression was markedly suppressed in the presence of PDTC at a concentration of 50 μM (Fig. 4A). The protein production of PAI-1 induced by Pg LPS was also greatly inhibited by the pretreatment with PDTC (Fig. 4B). These data suggest that PAI-1 production by Pg LPS is

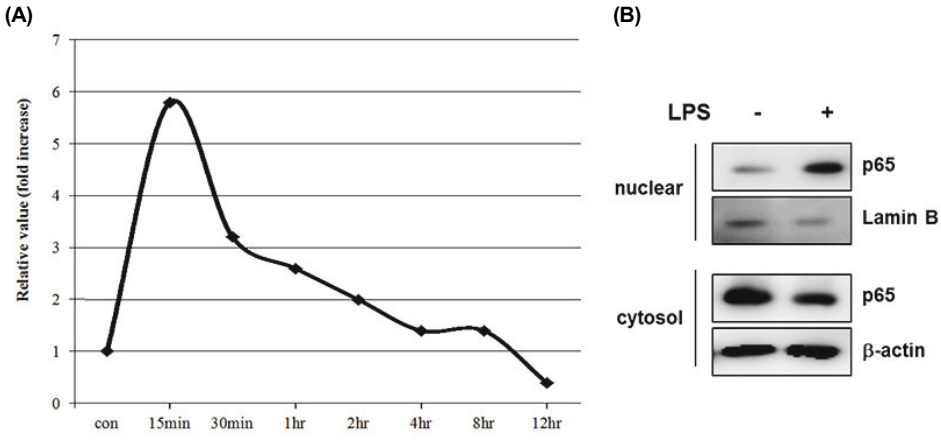

Fig. 3. (A) Activation of NF-κB in Pg LPS-treated HGF. The nuclear extracts were prepared from HGF after Pg LPS stimulation for various time intervals. NF-κB activation assay was carried out using an NF-κB p50/p65 Transcription Factor Assay. (B) HGF were treated with Pg-LPS (2 μg/ml) for 30 min, and the extracts were fractionated into the cytosol and nucleus. Successful fractionation was confirmed by Western blot using anti-β-actin antibody (cytosolic marker) and anti-Lamin B antibody (nuclear marker). NF-κB in the subcellular fractions was analyzed by Western blot using anti-p65 antibody.

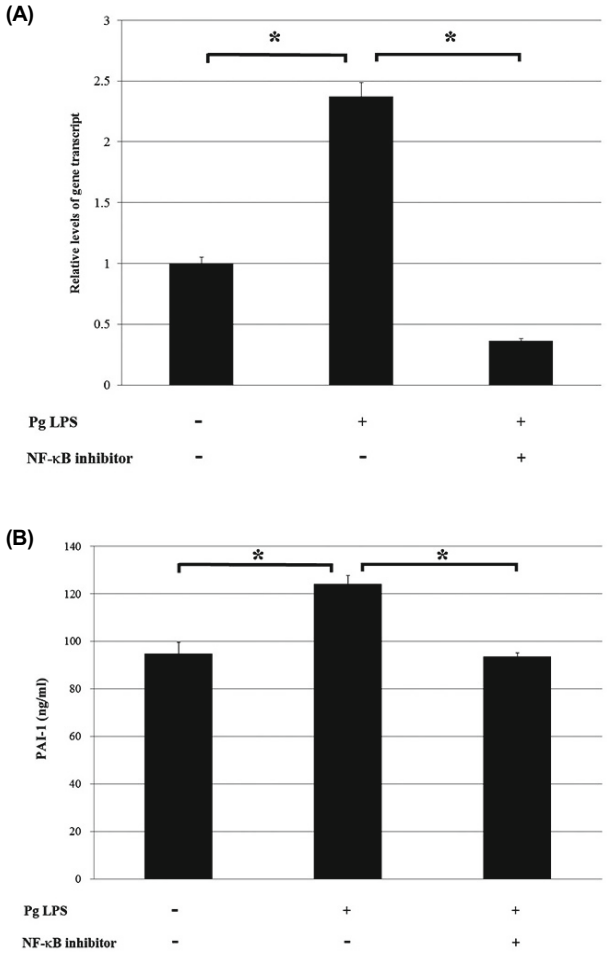

Fig. 4. Effects of PDTC on the Pg LPS-induced PAI-1 mRNA expression. (A) HGF were pretreated with PDTC (10 μM) for 30 min prior to Pg LPS treatment. Total RNA was prepared 30 min after Pg LPS treatment and real time-PCR was performed as described in 'Materials and Methods'. Effects of PDTC on the Pg LPS-induced PAI-1protein production. (B) HGF were pretreated with PDTC (10 μM) for 30 min prior to Pg LPS treatment, and the culture supernatants were collected 2 h after Pg LPS treatment. The levels of PAI-1production in culture supernatants were measured by ELISA. Values are expressed as mean±SD obtained from three independent experiments. * P<0.05.

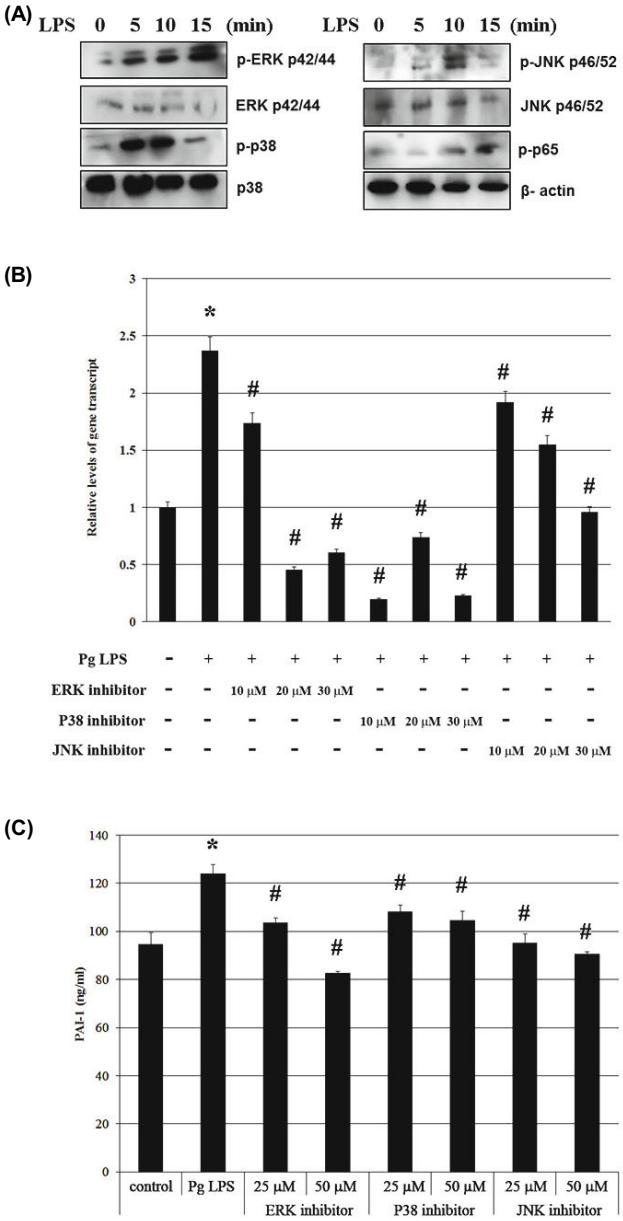

Fig. 5. Expression of MAK kinase and effects of MAP kinase inhibitors on the Pg LPS-induced PAI-1 mRNA expression. (A) HGF was treated with Pg-LPS (2 μg/ml) for the indicated time and cell lysates were subjected to Western blot for ERK, p38 and JNK detection. (B) HGF were pretreated with each MAP kinase inhibitors for 30 min prior to Pg LPS treatment. Total RNA was prepared 30 min after Pg LPS treatment and real time-PCR was performed as described in Materials and Methods. Effects of MAP kinase inhibitors on the Pg LPS-induced PAI-1 protein production. (C) HGF were pretreated with each MAP kinase inhibitors for 30 min prior to Pg LPS treatment, and the culture supernatants were collected 2 h after Pg LPS treatment. The levels of PAI-1 production in culture supernatants were measured by ELISA. Values are expressed as mean ± the SD obtained from three independent experiments. *P<0.05 control vs. Pg LPS; #P<0.05 Pg LPS vs. inhibitors.

mediated by NF-κB.

MAP kinase inhibitor suppressed Pg LPS-induced PAI-1 expression in HGF

To determine whether MAP kinase pathways are involved in PAI-1 up-regulation by Pg LPS, we examined whether Pg LPS activates MAP kinase using anti-phospho-MAP kinase antibody. Western blot analysis revealed that Pg LPS induced the phosphorylation of three MAP kinases at 5 min after LPS stimulation (Fig. 5A).

To further describe the role of MAP kinase in regulating PAI-1 expression induced by Pg LPS, HGF were pretreated with three MAP kinase inhibitors 30 min before Pg LPS stimulation at a concentration of 10, 20, or 30 μM. PD98059, SB203580, and SP600125 were used to inhibit ERK, p38 and JNK, respectively. All MAP kinase inhibitors suppressed PAI-1 gene expression induced by Pg LPS (Fig. 5B). The protein production of PAI-1 induced by Pg LPS was also inhibited by pretreatment with MAP kinase inhibitors (Fig. 5C). In sum, these findings suggest that all three MAP kinase pathways are involved in the PAI-1 expression by Pg LPS.

Discussion

In periodontal tissues, a number of genes are up- or down-regulated by subgingival plaque biofilm comprised of facultative or anaerobic bacteria such as *P. gingivalis*, *Tannerella forsythia*, and *Aggregatibacter actinomycetemcomitans* (Darveau, 2010). Inflammatory mediators including cytokines, chemokines, and their receptors are expressed by gingival fibroblast, epithelial cells, endothelial cells, and inflammatory cells in periodontal disease tissues (Eskan *et al.*, 2008; Gaibani *et al.*, 2010; Jonsson *et al.*, 2010). In our previous study, we used ACP system to screen for genes that were induced in periodontitis compared to the healthy gingiva (Na *et al.*, 2012). Among them, the increased expression of PAI-RBP1 was especially noticeable. PAI-RBP1 is a binding protein for PAI-1. Thus, we hypothesized that PAI-1 is associated with inflammatory response in periodontal tissue and may be induced by Pg LPS, a major pathogenic factor for periodontitis.

A primary role of PAI *in vivo* is the inhibition of both tissue- and urokinase-type plasminogen activators. In addition to this function, PAI-1 acts as an acute-phase protein during acute inflammation. The expression and secretion of PAI-1 by endothelial and a variety of other cells are strongly induced by a number of proinflammatory cytokines, and substantially increased PAI-1 levels are observed during inflammatory reactions (Levi *et al.*, 2003), acute lung injury (Idell, 2003), experimental sepsis (Saetre *et al.*, 2000), and sepsis (Sakata *et al.*, 1991). Increased PAI-1 levels have also been described in other disease entities, such as renovascular disease (Xu *et al.*, 1996), Kawasaki disease (Senzaki *et al.*, 2003), and asthma (Cho *et al.*, 2004). Consequently, increased PAI-1 levels are thought to be important in the pathogenesis of various inflammatory diseases. PAI-1 has been detected as well in inflamed gingiva (Xiao *et al.*, 1998; Kinnby *et al.*, 1999; Deppe *et al.*, 2010).

In this study, we tested whether Pg LPS induces PAI-1 expression *in vitro*. When an increasing concentration of Pg LPS was administered to HGF, PAI-1 mRNA expression increased in a dose-dependent manner. When HGF was challenged with Pg LPS over various time spans, the expression of PAI-1 mRNA increased 1 h after Pg LPs treatment and reached its maximum at 8 h. Pg LPS also induced high levels of PAI-1 protein production at 4 h and gradually increased the production of PAI-1 protein up to 24 h after Pg LPS challenge. Thus, Pg LPS induced both mRNA expression and protein production of PAI-1 in HGF. These results were supported by reports that PAI-1 was induced by LPS in epithelial cell and macrophage (Dos Santos *et al.*, 2007; Xu *et al.*, 2009).

NF-κB is a multiunit transcription factor that plays a central role in the induction of genes including proinflammatory cytokines and many other immunoregulatory genes (Ha *et al.*, 2009; Wang *et al.*, 2009; Wullaert *et al.*, 2010). However, the involvement of NF-κB in Pg LPS-induced PAI-1 expression has not been reported. Thus, we investigated whether NF-κB might be related to PAI-1 expression in HGF. Pg LPS rapidly induced the activation of NF-κB, reaching a maximum level at 15 min after Pg LPS treatment. Next, we used PDTC, NF-κB inhibitor to find out whether NF-κB activation is critical for PAI-1 expression. PAI-1 mRNA expression and protein synthesis were significantly suppressed by PDTC treatment, suggesting that Pg LPS induces PAI-1 expression through the activation of NF-κB.

MAP kinases pathway is central to many host responses and is a major signaling pathway, transmitting signals to immediate early genes implicated in the regulation of cytokine responses. Therefore, we also examined the possible involvement of MAP kinases in Pg LPS-induced PAI-1 expression. There are at least three main groups of MAP kinases, ERK, p38 and JNK, which can be activated in response to various extracellular stimuli in many types of cells (Tas *et al.*, 2005). In this study, using inhibitors of these MAP kinases, we have implicated ERK, p38 and JNK kinases in the induction of PAI-1 mRNA and protein by Pg LPS in HGF. These results allow us to hypothesize that PAI-1 induction is dependent on the ERK, p38 and JNK kinases signaling pathway. It has recently become clear that PAI-1 probably has other properties besides its inhibitory role in the fibrinolytic system. The exposure of mice to aerosolized LPS increased PAI-1 expression in the lung and alveolar compartment, which was decreased by pretreatment with the JNK inhibitor SP600125, suggesting the role for PAI-1 in the JNK-mediated pathway regulating LPS-induced neutrophil recruitment (Arndt *et al.*, 2005). Reliance of the NF-κB and MAP kinase pathways for PAI-1 expression was also demonstrated (Kruithof, 2008).

In summary, this study shows that Pg LPS induce PAI-1 expression that is regulated through NF-κB and MAP kinases pathway in HGF, suggesting the involvement of PAI-1 in periodontitis.

Acknowledgements

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