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Aggregatibacter actinomycetemcomitans Strongly Stimulates Endothelial Cells to Produce Monocyte Chemoattractant Protein-1 and Interleukin-8

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Aggregatibacter actinomycetemcomitans **is the most important etiologic agent of aggressive periodontitis and can interact with endothelial cells. Monocyte chemoattractant protein-1 (MCP-1) and interleukin-8 (IL-8) are chemokines, playing important roles in periodontal pathogenesis. In our current study, the effects of *A. actinomycetemcomitans* on the production of MCP-1 and IL-8 by human umbilical vein endothelial cells (HUVEC) were investigated. *A. actinomycetemcomitans* strongly induced the gene expression and protein release of both MCP-1 and IL-8 in a dose- and time-dependent manner. Dead *A. actinomycetemcomitans* cells were as effective as live bacteria in this induction. Treatment of HUVEC with cytochalasin D, an inhibitor of endocytosis, did not affect the mRNA up-regulation of MCP-1 and IL-8 by *A. actinomycetemcomitans*. However, genistein, an inhibitor of protein tyrosine kinases, substantially inhibited the MCP-1 and IL-8 production by *A. actinomycetemcomitans*, whereas pharmacological inhibition of each of three members of mitogen-activated protein (MAP) kinase family had little effect. Furthermore, gel shift assays showed that *A. actinomycetemcomitans* induces a biphasic activation (early at 1-2 h and late at 8-16 h) of nuclear factor-κB (NF-κB) and an early brief activation (0.5-2 h) of activator protein-1 (AP-1). Activation of canonical NF-κB pathway (IκB kinase activation and IκB-α degradation) was also demonstrated in these experiments. Although lipopolysaccharide from *A. actinomycetemcomitans* also induced NF-κB activation, this activation profile over time differed from that of live *A. actinomycetemcomitans*. These results suggest that the expression of MCP-1 and IL-8 is potently increased by *A. actinomycetemcomitans* in endothelial cells, and that the viability of *A. actinomycetemcomitans* and bacterial internalization are not required for this effect, whereas the activation of protein tyrosine kinase(s), NF-κB, and AP-1 appears to play important roles. The secretion of high levels of MCP-1 and IL-8 resulting from interactions of *A. actinomycetemcomitans* with endothelial cells may thus contribute to the pathogenesis of aggressive periodontitis.**

Key words: *Aggregatibacter actinomycetemcomitans*, monocyte chemoattractant protein-1, interleukin-8, endothelial cells

Introduction

Periodontitis, an inflammatory disorder of the supporting tissues of the teeth, results from the interplay between the specific subgingival microorganisms and the host's immune and inflammatory response [1,2]. Periodontitis can be categorized into three major forms: chronic, aggressive, and necrotizing forms [3]. Aggressive periodontitis comprises a

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group of rare, often severe, rapidly progressive forms of periodontitis often characterized by an early age of clinical manifestation and a distinctive tendency for cases to aggregate in families [4]. *Aggregatibacter actinomycetemcomitans* (formerly known as *Actinobacillus actinomycetemcomitans*) is a facultatively anaerobic nonmotile gram-negative rod, and evidence suggests that this species plays a primary etiological role in localized aggressive periodontitis. Some forms of chronic periodontitis are also found to be associated with *A. actinomycetemcomitans* [5]. The virulence factors of this pathogen include leukotoxins, immunosuppressive factors, and a high capacity to invade the host cells [6].

Whereas inflammation is a localized protective response elicited by injury and infection, excessive or sustained inflammation results in chronic inflammatory diseases such as periodontitis [7]. Pro-inflammatory cytokines and chemokines are key factors in the initiation and development of the inflammatory cascade. Chemokines selectively attract different cell types to the inflammatory site. Monocyte chemoattractant protein-1 (MCP-1) is one of the most potent chemoattractants for monocytes. Interleukin-8 (IL-8) displays a wide range of biologic effects, including chemotaxis and activation of neutrophils [8]. Endothelial cells produce both MCP-1 and IL-8 in response to various stimuli [9], and MCP-1 increases in the crevicular fluids of adult periodontal patients with severity of the disease [10]. Therefore, production of MCP-1 and IL-8 by bacterially stimulated gingival endothelial cells should contribute to periodontal disease progression.

Multiple signaling mechanisms have been reported to be involved in the intracellular activation of gene expression of MCP-1 and IL-8 in vascular endothelial cells by various stimuli [11,12]. A major mechanism through which signals from environmental stimuli are transmitted to the nucleus involves the activation of cellular kinases, including those belonging to the mitogen-activated protein (MAP) kinase superfamily. The MAP kinase group consists of three serine-threonine kinases: extracellular signal-regulated kinases (ERK) and the stress-activated protein kinases c-Jun N-terminal kinase (JNK) and p38 MAP kinase [13]. The promoter regions of genes of MCP-1 and IL-8 contain binding sites for the redox-responsive transcription factors activator protein-1 (AP-1) and nuclear factor-B (NF-κB), which have been shown to be important for expression of MCP-1 and IL-8 [14]. Cytokines are selectively expressed in a cell type-specific and stimulus-specific manner and the clarification of differential activation of intracellular signaling pathways provides a critical understanding for therapeutic intervention.

A. actinomycetemcomitans has been reported to modify host cytokine responses. Gingival epithelial cells could be stimulated to express IL-8 by *A. actinomycetemcomitans* [15]. Its lipopolysaccharide (LPS), lipid A-associated proteins and saline-extractable surface-associated material were reported to induce IL-6 in human gingival fibroblasts [16]. Also, mononuclear cells stimulated by *A. actinomycetemcomitans* produced MCP-1 and IL-8 [17]. However, there has been no report on the induction of MCP-1 and IL-8 by *A. actinomycetemcomitans* in endothelial cells. Therefore, the present study was performed to investigate the induction of MCP-1 and IL-8 in human umbilical vein endothelial cells (HUVEC) challenged with *A. actinomycetemcomitans*.

Materials and methods

Reagents

A. actinomycetemcomitans LPS was kindly provided by Professor Y.J. Kim (Department of Periodontology, Chonnam National University Dental School). Escherichia coli LPS and cytochalasin D were purchased from Sigma (St. Louis, MO, USA). Antibodies to phospho-IKKα/β (Ser176/180), IκB-α, phospho-p38 MAPK (Thr180/Tyr182), phospho-SAPK/JNK (Thr183/Tyr185) and phospho-ERK1/2 (Thr202/Tyr204) were from Cell Signaling Technology (Beverly, MA, USA). Anti-β-actin was from Sigma. Various inhibitors of signaling pathways were purchased from Calbiochem (Darmstadt, Germany). Unless stated otherwise, all chemicals were from Sigma.

Bacteria and growth conditions

A. actinomycetemcomitans ATCC 33384 was grown anaerobically (85% N_2, 10% H_2, and 5% CO_2) in Tryptic soy broth supplemented with yeast extract (1 mg/ml) and horse serum (10%) at 37C. Porphyromonas gingivalis 381 was grown anaerobically in Tryptic soy broth supplemented with yeast extract (1 mg/ml), hemin (5 μg/ml), and menadione (1 μg/ml). Bacteria in logarithmic growth phase

were used in all experiments. For the preparation of killed *A. actinomycetemcomitans*, bacteria were harvested, washed three times with phosphate-buffered saline (PBS), and resuspended in a small volume of PBS. The bacteria were then exposed to a standard germicidal ultraviolet lamp for 3 h and bacterial killing was confirmed by agar plating.

Human endothelial cell culture

HUVEC were purchased from Clonetics (Walkersville, MD, USA) and used between passages 3-7. The cells were cultured in Ham's F-12K medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen), 30 μg/ml endothelial cell growth supplement, 100 μg/ml heparin, and 50 μg/ml gentamicin at 37°C in 5% CO_2. Complete F-12K mediumwithout gentamicin was used for co-cultures of HUVEC and bacteria.

Infection protocol

Bacteria were washed twice with phosphate-buffered saline (PBS) and once with complete F-12K medium. After resuspension in complete F-12K 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. For enzyme-linked immunosorbent assays (ELISA), 5×10^4 HUVEC were seeded in 48-well plates. After 24 h, the medium was removed and the cells were incubated with various doses (multiplicity of infection (MOI) = 0.1-100) of live or killed bacteria in a final volume of 0.5 ml for 8 or 18 h. For Western blots, RT-PCR, and gel shift assays, 2×10^5 HUVEC were seeded in 6-well plates. After 24 h, the medium was removed and the cells were incubated with *A. actinomycetemcomitans* (MOI = 10) for various times.

Measurement of MCP-1 and IL-8 from culture supernatants

The HUVEC culture supernatants were collected, clarified, and the levels of MCP-1 and IL-8 were quantified using a commercial ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's directions.

Measurement of relative mRNA expression of MCP-1 and IL-8

Total RNA was prepared with Trizol reagent (Life Technologies) as specified by the manufacturer and quantified spectrophotometrically. Levels of MCP-1 and IL-8mRNA expression were determined by RT-PCR using specific primers. First-strand cDNA was synthesized from 1 μg of RNA using random primers (Promega, Madison, WI, USA) and Molony murine leukemia virus reverse transcriptase (Life Technologies). 2 μl of cDNA products was amplified in 25 μl volumes under a layer of mineral oil using a GeneAmp 9600 thermal cycler (Perkin Elmer Cetus, Norwalk, CT, USA). Each PCR reaction mixturecontained 50 mM KCl, 10 mM Tris-HCl, 1.5 μM $MgCl_2$, 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-TGTGCTCTCCAAATTTTTTTTACTG-3, 5- CTCTCTTTCCTCTTTAATGTCCAGC -3 for IL-8 (408 bp); and 5-AGCGGGAAATCGTGCGTG-3, 5-CAGGGTACATGGTGGTGCC-3 for β-actin (300 bp). The numbers of the PCR cycles for MCP-1, IL-8, and β-actin were 30, 30, and 28, respectively. The PCR products of 10 μl were fractionated on 2% agarose gels containing ethidium bromide, visualized by UV transillumination, and photographed.

Western blot

HUVEC in 6-well plates were scraped with 100 μl of Cell Lysis Buffer (Cell Signaling Technology). 20 g of each boiled sample was resolved by SDS-PAGE (10%) and transferred to a polyvinylidene difluoride membrane (Bio-Rad). The membrane was probed with a 1:1000 dilution of rabbit anti-phospho-IKK polyclonal antibody and a 1:2000 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-IκB-α (1:1000), anti-phospho-p38 MAPK (1:1000), anti-phospho-SAPK/JNK (1:1000), anti-phospho-ERK1/2 (1:1000) and anti-β-actin (1:5000).

Gel shift assay

Nuclear extracts were prepared from HUVEC according to the method of Dignam et al. with some modifications [18]. Briefly, 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 $MgCl_2$, 0.5 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 (Sigma), followed by vigorous vortex for 10 sec. 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 added 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) in a drop-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 the nuclear extracts were measured with DC Protein Assay Kit (Bio-Rad). Double-stranded NF-κB (5'-AGTTGAGGGGACTTTCCCAGGC-3')- and AP-1 (5'-CGCTTGATGAGTCAGCCGGAA-3')-binding DNA probes were purchased from Promega, and end-labeled with [γ-^{32}P] ATP and T4 polynucleotide kinase. 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 30min at room temperature. The DNA-protein complexes were separated on 4% polyacrylamide gels and visualized by autoradiography.

Results

Production of MCP-1 and IL-8 protein by *A. actinomycetemcomitans* in HUVEC

HUVEC were incubated with increasing doses of live *A. actinomycetemcomitans* for 18 h, and concentrations of MCP-1 and IL-8 in the collected supernatants were measured by ELISA. As shown in Fig. 1, increased concentrations of both chemokines were obtained with increasing bacterial doses. The fold increases by the highest dose (MOI = 100) of *A. actinomycetemcomitans* were about 5 and 10 for MCP-1 and IL-8, respectively. It was notable that MCP-1 release was substantially increasedeven with a very low MOI challenge (MOI=0.1). Next, the time course of the chemokine release was examined. HUVEC were challenged with *A. actinomycetemcomitans* at an MOI of 10 for up to 32 h, and the production of MCP-1 and IL-8 was measured every four hours. The overall kinetics of both MCP-1 and IL-8 release were similar. The amount of MCP-1 and IL-8 release was significantly enhanced after 4 h of stimulation and continued to increase up to 16 h, after which the chemokine levels were maintained up to 32 h (Fig. 2).

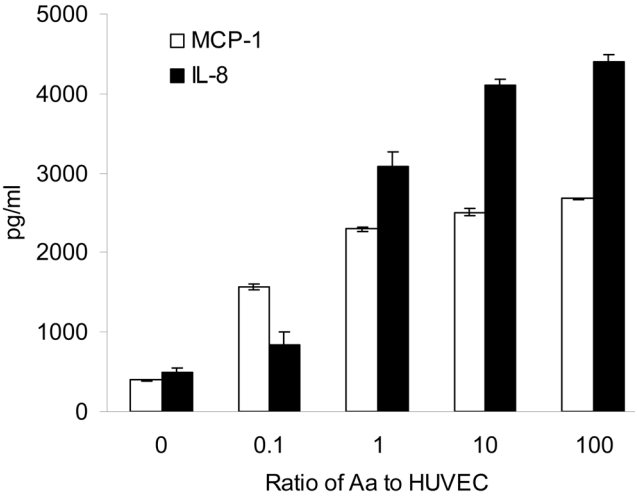

Fig. 1. Induction of MCP-1 and IL-8 protein release by *A. actinomycetemcomitans* in endothelial cells. 5×10^4 HUVEC were seeded in 48-well plates. After 24 h, the cells were incubated with different doses (MOI = 0.1-100) of *A. actinomycetemcomitans* in a final volume of 0.5 ml for 18 h. Data are the mean±S.D. of a representative experiment performed in triplicate. Similar results were obtained in two other experiments.

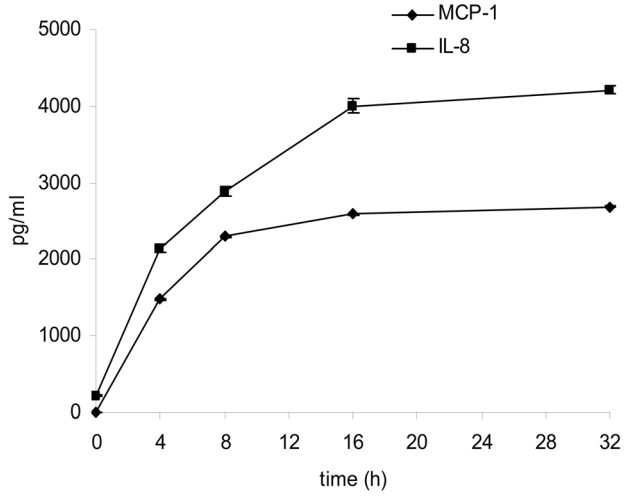

Fig. 2. Time course of *A. actinomycetemcomitans*-induced production of MCP-1 and IL-8 in endothelial cells. 5×10^4 HUVEC were stimulated with *A. actinomycetemcomitans* at an MOI of 10 in a final volume of 0.5 ml. Culture supernatants were collected every 4 hours up to 32 h after bacterial challenge. MCP-1 and IL-8 concentrations of the culture supernatants were measured by ELISA. Data are the mean± S.D. of a representative experiment performed in triplicate. Similar results were obtained in two other experiments.

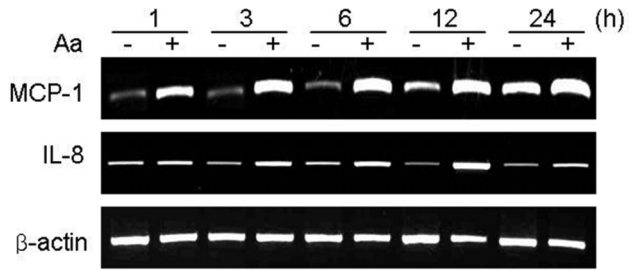

Fig. 3. mRNA induction of MCP-1 and IL-8 by *A. actinomycetemcomitans* in endothelial cells. 2×10^5 HUVEC were seeded in 6-well plates. After 24 h, the cells were stimulated with *A. actinomycetemcomitansat* an MOI of 10 in a final volume of 2 ml. Total RNA was isolated at 1, 3, 6, 12, and 24 h after bacterial challenge, and levels of MCP-1 and IL-8 mRNA were determined by RT-PCR. Shown are photographs of agarose gels from one experiment, representative of three.

mRNA expression of MCP-1 and IL-8

In order to examine the induction of MCP-1 and IL-8 at the steady-state mRNA level, RT-PCR analysis was performed on RNA extracted from HUVEC that had been stimulated by *A. actinomycetemcomitans* (MOI = 10) for 1, 3, 6, 12 and 24 h (Fig. 3). The steady-state levels of MCP-1 mRNA expression in HUVEC were increased with *A. actinomycetemcomitans* infection throughout a 24-h time period. The MCP-1 mRNA levels were increased as early as 1 h after *A. actinomycetemcomitans* infection. Meanwhile, IL-8 mRNA levels reached peak levels at 12 h and declined near the baseline levelsat 24 h. Expression of MCP-1, but not IL-8, increased significantly over time even without bacterial stimulation. This is likely due to higher levels of spontaneous production of MCP-1.

Comparison between live and killed *A. actinomycetemcomitans*

To determine if differential response of MCP-1 and IL-8 induction occurs according to bacterial viability, HUVEC cultures were incubated with the same number of live or killed *A. actinomycetemcomitans* (MOI = 10)for 18 h. The ELISA results showed that both live and UV-killed *A. actinomycetemcomitans* similarly increased MCP-1 and IL-8 secretion (Fig. 4).

Role of bacterial internalization

It was of interest to determine whether internalization of bacteria is necessary or adhesion itself is sufficient for MCP-1 and IL-8 induction by *A. actinomycetemcomitans* in HUVEC. For this purpose, HUVEC were treated with

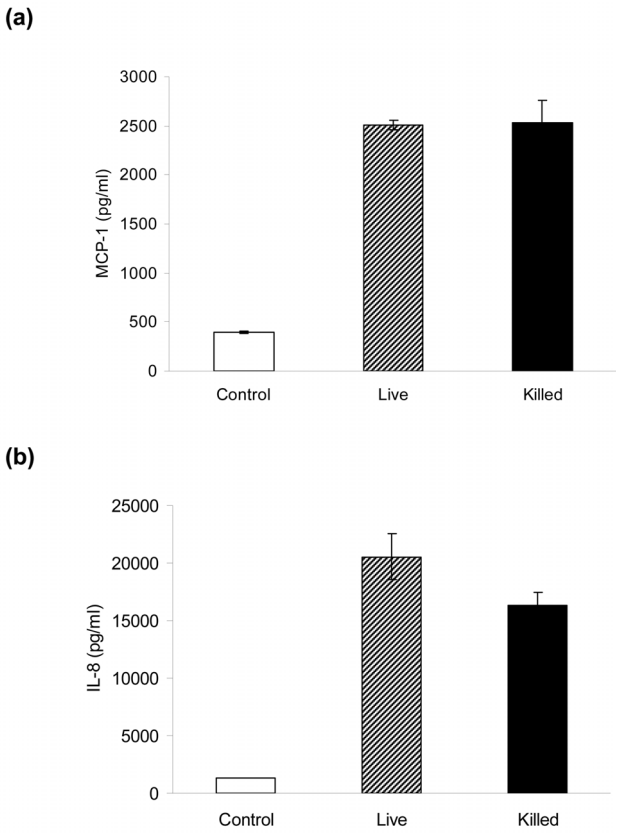

Fig. 4. Comparison between live and killed *A. actinomycetemcomitans* for MCP-1 and IL-8 production by endothelial cells. 5×10^4 HUVEC were seeded in 48-well plates. After 24 h, the cells were incubated with live or UV-killed *A. actinomycetemcomitans* at an MOI of 10 in a final volume of 0.5 ml for 18 h. MCP-1 (a) and IL-8 (b) concentrations of the culture supernatants were measured by ELISA. Data are the mean± S.D. of a representative experiment performed in triplicate. Similar results were obtained in two other experiments.

cytochalasin D, an inhibitorof endocytosis. Cytochalasin D, acting as an inhibitor of actin polymerization, prevents uptake of bacteria without interfering with the binding of bacteria. Effect of bafilomycin A1, an inhibitor of endosomal maturation, was also observed. In parallel, *Porphyromonas gingivalis* was used and compared because P. gingivalishas been known to induce MCP-1 in a bacterial internalization-dependent manner [19]. Treatment of Cytochalasin D (1 μg/ml) or bafilomycin A1 (100 nM) did not affect *A. actinomycetemcomitans*-induced MCP-1 and IL-8 mRNA expression (Fig. 5). As expected, *Escherichia coli* LPS-induced responses were not inhibited by cytochalasin D, and consistent with a previous report, cytochalasin D prevented MCP-1 mRNA induction by *P. gingivalis*.

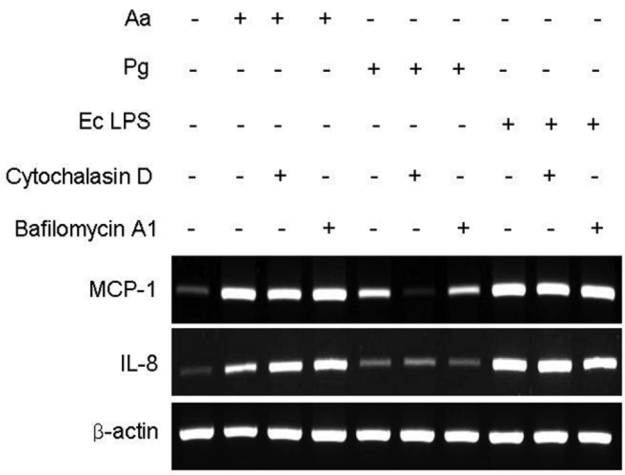

Fig. 5. Effect of cytochalasin D and bafilomycin A1 on MCP-1 and IL-8 mRNA expression in endothelial cells. 2×10^5 HUVEC were seeded in 6-well plates. After 24 h, the cells were pre-treated with cytochalasin D (1 μg/ml) or bafilomycin A1 (100 nM) for 30 min. Then the cells were stimulated with *A. actinomycetemcomitans* (MOI=1:10), *P. gingivalis* (MOI=1:100), or *E. coli* LPS (0.1 μg/ml) in a final volume of 2 ml for 3 h. Total RNA was extracted and expression of MCP-1 and IL-8 mRNA was determined by RT-PCR. Shown are photographs of agarose gels from one experiment, representative of three.

Effect of various signaling inhibitors

The MAP kinases are central to many inflammatory signaling pathways. In order to evaluate the relative importance of MAP kinases and some additional signaling pathways in *A. actinomycetemcomitans*-induced chemokine-sexpression in endothelial cells, specific pharmacological inhibitors were used. HUVEC were pretreated with GF109203X (protein kinase C, 1 μM), wortmannin (phosphatidylinositol 3-kinase, 100 nM), U73122 (phospholipase C, 10 μM), genistein (protein tyrosine kinase, 50 μM), PD98059 (ERK, 50 μM), SB203580 (p38 MAP kinase, 10 μM), SP600125 (JNK, 10 μM), or rapamycin (mammalian target of rapamycin, 10 nM) for 1 h, and then HUVEC were incubated with *A. actinomycetemcomitans* for 8 h. MCP-1 and IL-8 concentrations of the collected supernatants were measured by ELISA. Unexpectedly, SB203580 and SP600125, inhibitors of p38 MAP kinase and JNK respectively, only slightly reduced MCP-1 and IL-8 levels (Fig. 6). Instead, genistein, an inhibitor of protein tyrosine kinase most strongly inhibited the MCP-1 and IL-8 production by *A. actinomycetemcomitans*, resulting in a half reduction.

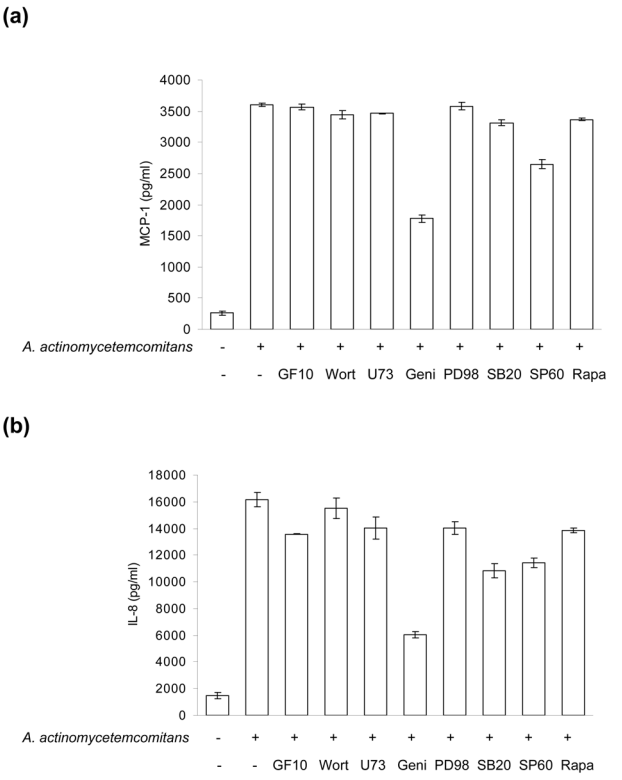

Fig. 6. Effect of various signaling inhibitors on MCP-1 and IL-8 production by *A. actinomycetemcomitans* in endothelial cells. 5×10^4 HUVEC were seeded in 48-well plates. After 24 h, the cells were pretreated for 1 h with GF109203X (1 μM), wortmannin (100 nM), U73122 (10 μM), genistein (50 μM), PD98059 (50 μM), SB203580 (10 μM), SP600125 (10 μM), or rapamycin (10 nM), and then stimulated with *A. actinomycetemcomitans* in a final volume of 0.5 ml for 8 h. MCP-1 (a) and IL-8 (b) concentrations of the culture supernatants were measured by ELISA. Data are the mean± S.D. of a representative experiment performed in triplicate. Similar results were obtained in two other experiments.

Activation of transcription factors NF-κB and AP-1

Since NF-κB and AP-1 are the two most important transcription factors for both MCP-1 and IL-8 gene expression, activation of NF-κB and AP-1 in *A. actinomycetemcomitans*-exposed HUVEC was analyzed by gel shift assays. HUVEC were incubated with *A. actinomycetemcomitans* (MOI = 10) for 0.5-24 h. Since *A. actinomycetemcomitans* LPS is known as a potent inducer of cytokines in various cells, its activity for activation of NF-κB and AP-1 was also observed in parallel experiments. As shown in Fig. 7a, inducible NF-κB and AP-1 DNA binding activities were detected after stimulation with *A. actinomycetemcomitans*. *A. actinomycetemcomitans* induced a biphasic activation (early at 1-2 h and late at 8-16 h) of

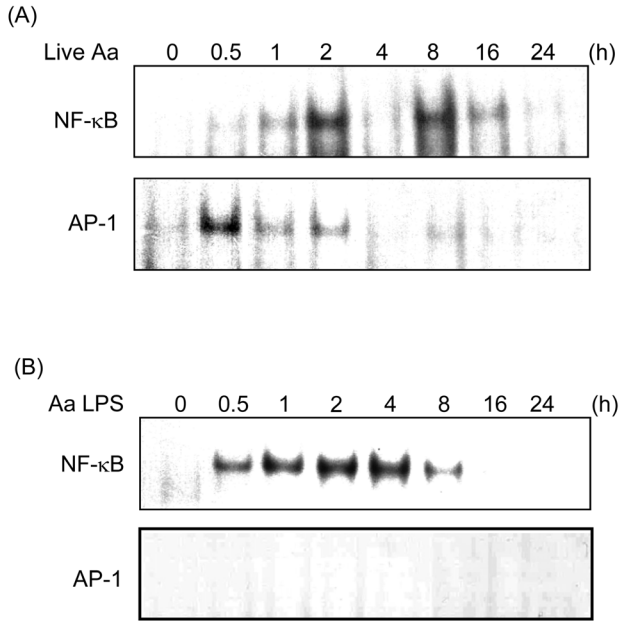

Fig. 7. Activation of NF-κB and AP-1 by *A. actinomycetemcomitans* in endothelial cells. 2×10^5 HUVEC were seeded in 6-well plates. After 24 h, the cells were incubated with *A. actinomycetemcomitans* (MOI = 10) or *A. actinomycetemcomitans* LPS (0.1 μg/ml) for the indicated time periods. Nuclear extracts were prepared and activation of NF-κB and AP-1 was determined by gel shift assays. One of three experiments with similar results is shown.

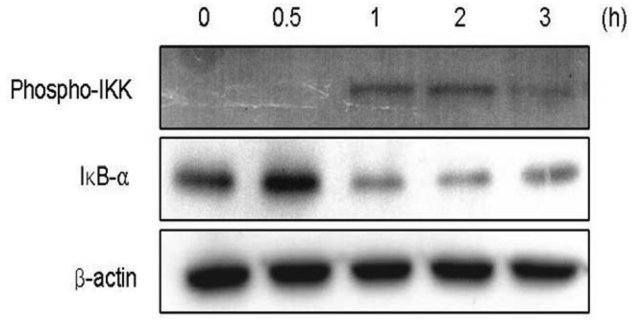

Fig. 8. Activation of IKK and degradation of IκB-α by *A. actinomycetemcomitans* in endothelial cells. 2×10^5 HUVEC were seeded in 6-well plates. After 24 h, the cells were challenged with *A. actinomycetemcomitans* (MOI = 10) for the indicated time periods. Cell lysates were prepared and Western blot analysis was performed for phospho-IKK and IκB-α. One of three experiments with similar results is shown.

NF-κB and a early brief activation (0.5-2 h) of AP-1. Although LPS from *A. actinomycetemcomitans* (0.1 g/ml) also induced NF-κB activation, the activation profile over time course differed from that by live *A. actinomycetemcomitans*. *A. actinomycetemcomitans* LPS did not induce an AP-1 DNA binding activity (Fig. 7b).

Activation of IKK

IKK activation and subsequent IκB degradation are the canonical pathway for NF-κB activation in response to a given stimulus. To determine whether *A. actinomycetemcomitans* stimulates the canonical pathway, leading to NF-κB activation, HUVEC were challenged with *A. actinomycetemcomitans* (MOI = 10) for 0.5, 1, 2, and 3 h, and Western blot analysis was performed for phospho-IKK and IκB-α. Phosphorylation of IKK and degradation of IκB-α by *A. actinomycetemcomitans* were demonstrated, in which IKK phosphorylation began at 1 h and was maintained up to 3 h after stimulation (Fig. 8).

Discussion

Chemokines, which are responsible for the recruitment of leukocytes, arecrucial mediators of inflammation. Endothelial cells produce chemokines in response to inflammatory cytokines and microbial pathogens. In the present study, *A. actinomycetemcomitans* stimulated strongly and rapidly the release of both MCP-1 and IL-8 in HUVEC. The strong induction of MCP-1 and IL-8 release by *A. actinomycetemcomitans* is probably attributable to the robust and prolonged mRNA expression (Fig. 3). It was notable that substantial amounts of both chemokines were secreted with relatively low doses (MOI = 0.1-1) of *A. actinomycetemcomitans*. *A. actinomycetemcomitans* can adhere to and invade endothelial cells [20]. Interactions of endothelial cells with periodontopathogens should take place with high frequencies in highly vascularized periodontal tissues. In periodontal tissues, interactions of *A. actinomycetemcomitans* with endothleial cells could increase MCP-1 and IL-8 concentrations at local environments, which will attract monocytes and neutrophils, respectively, contributing to the pathogenesis of periodontitis.

Killed *A. actinomycetemcomitans* was as effective as live bacteria for MCP-1 and IL-8 induction. Moreover, bacterial internalization was not necessary for MCP-1 and IL-8 mRNA up-regulation by *A. actinomycetemcomitans*. These data suggest that HUVEC can be sufficiently stimulated by adhesion of *A. actinomycetemcomitans* without the need of bacterial internalization. This is in sharp contrast to the MCP-1 induction by *P. gingivalis*. *P. gingivalis*-induced

MCP-1 induction in HUVEC required bacterial internalization and live *P. gingivalis* was more effective than killed bacteria for MCP-1 induction [19,21]. The difference between the two important periodotopathogens regarding chemokines induction in endothelial cells is not explainable at present.

In this study, MAP kinase pathways were not involved in MCP-1 and IL-8 production by *A. actinomycetemcomitans*, which is also in contrast to the MCP-1 induction by *P. gingivalis*. Inhibitors of p38 MAP kinase and JNK significantly attenuated MCP-1 production by *P. gingivlais.* Therefore, endothelial cells are differentially stimulated by *A. actinomycetemcomitans* and *P. gingivalis* in many aspects. Among various signaling inhibitors, only genistein (a protein tyrosine kinase inhibitor) substantially inhibited the MCP-1 and IL-8 production by *A. actinomycetemcomitans*, suggesting an important role of a protein tyrosine kinase for endothelial cell stimulation by *A. actinomycetemcomitans*. However, further study is needed for elucidation of this pathway in *A. actinomycetemcomitans*-induced MCP-1/IL-8 production in endothelial cells.

Activation of various signaling pathways ultimately leads to activation of transcription factors for expression of pertinent genes. The 5′ flanking regions of the MCP-1 and IL-8 genes contain multiple 12-O-tetradecanoylphorbol-13-acetate response elements (TRE) and κB sites, suggesting potential roles of AP-1 and NF-κB. Inflammatory cytokines, including IL-1 and TNF-α, activate NF-κB and AP-1 and induce the expression of the MCP-1 and IL-8 genes in endothelial cells [14,22]. The canonical pathway for activation of NF-κB consists of IKK activation and subsequent degradation of IκB-α. Removal of IκB-α from NF-κB allows NF-κB to translocate into the nucleus [23]. AP-1 factor is composed of the proteins Fos and Jun. Activation of AP-1 typically involves synthesis of the Fos protein and phosphorylation of preexisting Jun protein. Transcription and synthesis of Fos can be enhanced by the ERK pathway. JNK phosphorylates c-Jun, and AP-1 complexes containing the phosphorylated form of Jun have increased transcriptional enhancing activity [24]. The induction of MCP-1 mRNA expression by *P. gingivalis* has been reported to involve both NF-κB and AP-1 transcription factors. Induction of MCP-1 by other infectious pathogens such as *Chlamydia pneumoniae* or *Borrelia burgdoferi* also requires the activation of NF-κB in endothelial cells [25,26]. Meanwhile, MCP-1 induction by Orientia tsutsugamushi is independent of NF-κB and is mediated by AP-1 activation [27]. In this study, activation of NF-κB and AP-1 was observed in *A. actinomycetemcomitans*-stimulated HUVEC. These results indicate that *A. actinomycetemcomitans* activates both NF-κB and AP-1 in endothelial cells and the combinational action of the two transcription factors results in enhanced transcription of MCP-1 and IL-8 genes.

Although *A. actinomycetemcomitans* LPS is a well-known inducer of various cytokines, the chemokines induction by *A. actinomycetemcomitans* is not most likely to be mediated by its LPS. If the LPS component of *A. actinomycetemcomitans* had mostly mediated the induction, the activation profile of NF-κB activation over time course should have been similar to that by live *A. actinomycetemcomitans*. Furthermore, unlike the whole cells of *A. actinomycetemcomitans*, its LPS did not induce an AP-1 DNA binding activity (Fig. 7). Therefore, the strong MCP-1/IL-8-inducing activity of live *A. actinomycetemcomitans* cannot be attributed entirely to its LPS.

To summarize, endothelial cells produce high levels of MCP-1 and IL-8 in response to *A. actinomycetemcomitans*, which was independent of viability and cellular uptake of *A. actinomycetemcomitans*. The underlying signaling mechanisms appear to include activation of a protein tyrosine kinase, IKK, NF-κB, and AP-1.

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