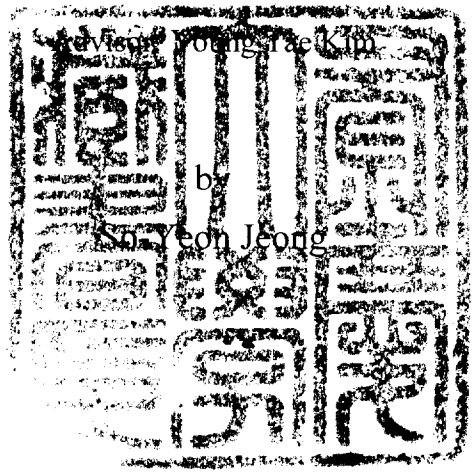
Nuclear Factor-κB activation induced by *Actinobacillus actinomycetemcomitans* Lipopolysaccharide

*Actinobacillus actinomycetemcomitans*의 Lipopolysaccharide에 의해 유도되는 Nuclear Factor-κB의 활성



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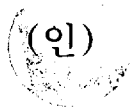
위 원 이학박사 이 명 숙 

위 원 이학박사 김 영 태 

Nuclear Factor-κB activation induced

By *Actinobacillus actinomycetemcomitans* Lipopolysaccharide

A dissertation

by

So-Yeon Jeong

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Contents

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ABSTRACT

Actinobacillus actinomycetemcomitans is a Gram-negative capnophilic rod and is associated with localized aggressive periodontitis, a disease characterized by rapid loss of the alveolar bone surrounding the teeth. Lipopolysaccharide (LPS), one of the virulent factors isolated from this periodontal pathogenic bacterium, has been shown to stimulate bone resorption. *A. actinomycetemcomitans* LPS may play a crucial role in periodontal tissue destruction during the course of aggressive inflammatory periodontal disease.

In this study, I investigated that *A. actinomycetemcomitans* LPS mediated NF-κB activation and the expression of both TNF-α and IL-1β genes by its activation using microarray, RT-PCR, ELISA and Western blot. In the experiments, the results showed that *A. actinomycetemcomitans* LPS can leads to the expression of NF-κB-related genes at 2hr, 18hr and enhanced the mRNA expression and protein synthesis of inflammatory cytokine, TNF-α and IL-1β, in RAW 264.7 cells. Western blotting analysis showed that *A. actinomycetemcomitans* LPS activated NF-κB in the nuclear protein. Furthermore, *A. actinomycetemcomitans* LPS-induced inflammatory cytokine, TNF-α and IL-1β, were shown to be suppressed by the pyrrolidine dithiocarbamate, an NF-κB inhibitor.

The results indicated that *A. actinomycetemcomitans* LPS has an effect on localized aggressive periodontitis for induction of inflammatory cytokine, TNF-α and IL-1β, through the activation of NF- κB.

INTRODUCTION

Actinobacillus actinomycetemcomitans (*A. actinomycetemcomitans)* is a gram-negative bacterium that has been identified in several human infectious diseases, such as endocarditis, meningitis, osteomyelitis, and aggressive periodontitis. The localized aggressive periodontitis destroyed by the bacterium is an early-onset form of disease and the periodontal destruction is most apparent around the first molars and incisor (Kesavalu *et al*, 2002).

Lipopolysaccharide (LPS) is a major component of the outer membrane of gram-negative bacteria and has been shown to stimulate bone resorption, epithelial cell destruction and proinflammatory cytokine production from monocytes/macrophages and neutrophils (Coil *et al*, 2004). Pro-inflammatory cytokines such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α have been reported to initiate and argument subsequent inflammatory cascades leading to tissue destruction.

A. actinomycetemcomitans Lipopolysaccharide (LPS) is a one of the virulent factors isolated from this periodontal pathogenic bacterium. It may play a crucial role in periodontal tissue destruction during the course of aggresive inflammatory periodontal disease (Sosroseno *et al*, 2002).

The innate immune response is initiated by recognition of microbial surface or secreted components. LPS is recognized by the Toll-like receptors (TLRs) present on immune cells and has been identified as the initial signaling event in the response to LPS. TLRs have been shown to activate IL-IR-assiciated kinase (IRAK) via an adaptor protein myeloid differentiation factor 88 (MyD88). Subsequent to the activation of IRAK, another adaptor protein TNF receptor-associated factor (TRAF) 6 is phosphorylated and recruited to IRAK. Nuclear factor (NF)-κB associates with inhibitory κB (IκB) proteins, which sequester NF-κB in the cytoplasm. To release NF-κB from the inhibition of IκB, the phosphorylation of IκB by IκB kinases (IKKs) is required. The TRAF6 is activated IKK complex through two pathway, NF-κB-inducing kinase (NIK) and evolutionarily conserved signaling intermediate in Toll pathways (ECSIT). The IκB is phosphorylated by IKK complex, and is degraded by proteasome. The separated NF-κB is translocated into the nucleus and activated (Takeda *et al*, 2004). NF-κB regulates expression of multiple genes which are involved in immunological and inflammatory responses (Figure 1). NF-κB regulated the genes such as MHC molecules, cytokines, growth factors and their receptors (TNF- α, IL-1β, IL-2, IL-6, IL-8, IL-12, G-CSF) and cell adhesion molecules (ICAM-1, VCAM-1, and E-selectin).

The microarray technology has provided a new and powerful tool that allows the simultaneous analysis of a large number of nucleic acid hybridizations in a rapid and efficient fashion. This technology is extremely useful in understanding the interrelationship of gene expression involved in the immune systems (Baiteng *et al*, 2001).

In this study, I have investigated that *A. actinomycetemcomitans* LPS mediated NF-κB activation and the expression of both TNF-α and IL-1β genes by its activation.

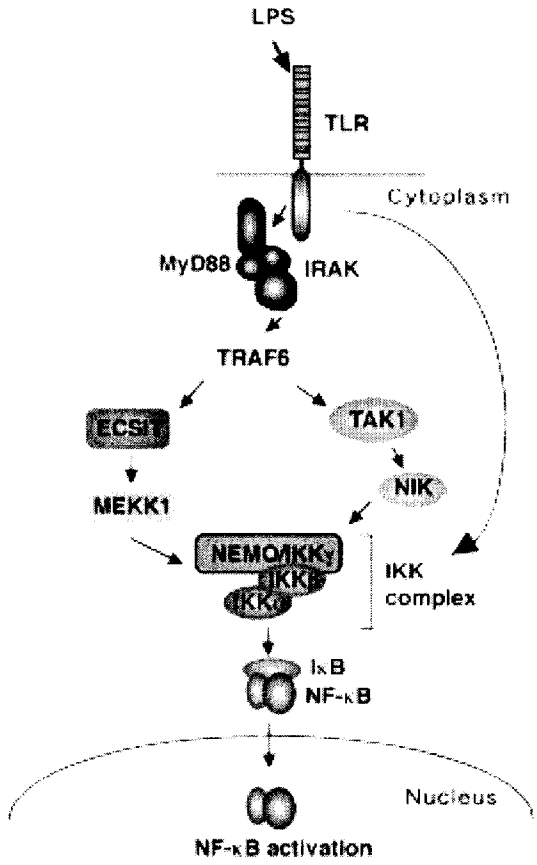

Figure 1. Mechanisms of NF-κB signaling pathway induced by LPS (Takeda *et al*, 2004).

MATERIALS AND METHODS

Reagents

The mouse monocyte-macrophage cell line (RAW264.7) was purchased from American Type Culture Collection (Rockville, MD. USA). Signal Transduction PathwayFinder Gearray^{TM} kit was obtained from Superarray Bioscience Corp (Frederick, MD. USA). Mouse TNF and IL-1β ELISA kit were purchased from R&D Systems (Minneapolis, MN. USA).

Bacteria and culture conditions

Actinobacillus actinomycetemcomitans was cultured in a tryptic soy broth, which contained 5 mg/ml hemin and 0.5 mg/ml of vitamin K at 37 ℃ in an anaerobic chamber with an atmosphere containing 90 % N_2, 5 % H_2 and 5 % CO_2.

LPS purification

A. actinomycetemcomitans was grown under the anaerobic conditions and then harvested at the end of the logarithmic phase of growth. LPS extraction was achieved by the hot-phenol-water method. Briefly, bacterial cell pellet was suspended in pyrogen-free water, and then equal volume of 90 % phenol pre-warmed at 60 ℃ was added dropwise for 20 min and stirred constantly. The aqueous phase was separated by centrifugation at 7,000 rpm for 15 min at 4 ℃ and collected. This process was repeated, and the aqueous phase was pooled and dialyzed against deionzed water for 3 days at 4 ℃. The dialyzed LPS preparation was then centrifuged at 40,000 rpm for 1.5 h at 4 ℃ using a Beckman Ultracentrifuge (Palo Alto, CA. USA). The precipitate was suspended with 30 ml of pyrogen-free water, dialyzed against distilled water for 3 days, lyophilized and stored at 4 ℃. LPS samples were separated by SDS-PAGE and stained by Coomassie brilliant blue R-250 to confirm the purity of the LPS moieties.

Cell culture

RAW264.7 cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10 % FBS (Life Technologies Inc., Paisley, Scotland), 100 U/ml penicillin and 100 ㎍/ml of streptomycin, and were incubated at 37 ℃ in a humidified atmosphere of 5 % CO_2.

RNA preparation

RAW 264.7 cells were treated for 2 h and 18 h with 100 ng/ml of A. actinomycetemcomitans LPS. Total RNA from these cells was isolated using TRIzol™ reagent (Invitrogen, Carlsbad, CA). The cells were directly lysed by adding 1 ml of TRIzol™ reagent in a culture dish. Following sample lysis for 5 min, the lysates were transfered to a new vial. 0.2 ml of chloroform was added into the lysates and shaken vigorously by hand for 15 sec followed by incubation for 2 to 3 min at room temperature. After centrifugation at 12,000 x g for 15 min, the aqueous phase was transferred to a fresh tube. The RNA from the aqueous phase was precipitated by mixing with isopropyl alcohol. After adding 0.5 ml of isopropyl alcohol, the sample was incubated for 10 min

and centrifuged at 12,000 x g for 10 min. The supernant was removed and the pellet was washed with 75 % ethanol. At the end of the procedure, the RNA pellet was dried and dissolved in RNase-free water.

Microarray

The microarray analysis was performed using the nonradioactive signal transduction pathway finder GEArray Q series kit (SuperArray Inc., MD. USA) according to the manufacturer's instructions. The array membrane is composed of 96 signal transduction pathway gene. The plasmid pUC18 as a negative control, and four housekeeping genes including glyceraldehydes-3-phosphate dehydrogenase (GAPDH), cyclophilin A, ribosomal protein L13a, and β-actin, each gene is printed with tetra spots format. The biotin-16-dUTP-labeled cDNA probes were synthesized from 5 ㎍ of total RNA. After pre-hybridization with GEAhyb Hybridization solution (SuperArray Inc., MD. USA) containing 100 ㎍/ml of denatured salmon sperm DNA (Invitrogen, CA. USA) for 2 h at 60℃, the array membrane was hybridized with denatured cDNA probed at 60 ℃ for overnight. Following washing the membrane twice with 2 x SSC, 1 % SDS and twice with 0.1 x SSC, 0.5 % SDS for 15 min at

60 ℃ each, the membrane was blocked with GEAblocking Solution Q (SuperArray Inc., MD. USA) for 40 min and incubated with alkaline phosphatase-conjugated for 10 min at room temperature. Chemiluminescent detection was performed using CDP-Star chemiluminescent substrate and the array image was recorded with X-ray film. The image was scanned with a scanner TouchToss SIS-3800 (Samsung, Seoul, Korea). The resulting scanned image was converted to a raw data file using Scanalyse software. GEArray Analyzer software (SuperArray Inc., MD. USA) was used for data analysis. The relative expression levels of different genes were estimated by comparing its signal intensity with that of internal control of β-actin.

RT-PCR (Reverse Transcriptase-Polymerase Chain Reaction)

Total RNA was prepared from RAW264.7 cells using TRIzol™ reagent according to the manufacturer's instructions. The concentration of the RNA obtained was determined by measuring the absorbance at 260 and 280 nm. RT-PCR was performed using a commercial premix kit (Bioneer, Daejeon, Korea) and the reaction mixture contained total 1 μg of RNA, each 25 pmol of specific sense and anti-sense primers in a thermocycler (PerkinElmer 2400). cDNA was amplified by PCR in a thermocycler (denaturation for 30 sec at

95 ℃, annealing for 30 sec at 60 ℃ and elongation for 30 sec at 72 ℃) using TNF-α (25 cycles), IL-1β (28 cycles) or β-action primers (35 cycles). The primers used in these analysis are as follows : TNF : 5'CCT GAT GCC CAC GTC GTA GC3' and 5'TTG ACC TCA GCG CTG AGT TG3'; IL-1β: 5'GAT ACA AAC TGA TGA AGC TCG TCA3' and 5'GAG ATA GTG TTT GTC CAC ATC CTG A3'; β-actin : 5'GGG TCA GAA CTC CTA TG3' and 5'GTA ACA ATG CCA TGT TCA AT3'; After reaction, fifteen microliters of the reaction products were analyzed on 1.2 % (w/v) agarose gel electrophoresis and stained with ethidium bromide (EtBr).

ELISA (Enzyme Linked Immunosorbent Assay)

The protein levels of TNF and IL-1β released to the culture media after *A. actinomycetemcomitans* LPS stimulation were analyzed by using an ELISA kit from R&D systems (Mineapolis, MN. USA). Briefly, control, standard or sample solution was added to ELISA well plate, which had been pre-coated with a specific monoclonal capture antibody. After incubating for 2 h at room temperature, polyclonal anti-TNF and anti-IL-1β antibody conjugated with horseradish peroxidase was added to the solution, and incubated for 2 h at

room temperature. Substrate solution containing hydrogen peroxidase and chromogen was added and allowed to react for 30 min. The levels of cytokines were measured by an ELISA reader at 450 nm. Each densitometric value expressed as mean ± SD was obtained from three independent experiments.

Extraction of the cytosolic and the nuclear protein

The nuclear extracts from RAW 264.7 cells were prepared after *A. actinomycetemcomitans* LPS stimulation. Briefly, cells were washed with ice-cold Phosphate Buffered Saline (PBS) and pelleted. The cell pellet was resuspended in Hypotonic buffer [1.5 mM $MgCl_2$, 1 mM DTT, 0.1 % NP-40, 20 mM β-glycerophosphate, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 10 mM Tris-HCl (pH 8.0), 0.1 % pepstatin, 0.1 % aprotinin] and incubated for 15 min on ice followed by vigorous voltex for 10 sec. The lysates were centrifuged, and supernatants were transferred to new vials. The pellet was resuspended in Extract buffer [10 mM Tris-HCl (pH 8.0), 50 mM KCl, 300 mM NaCl, 1 mM DTT, 20 mM β-glycerophosphate, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 0.1 % pepstatin, 0.1 %

aprotinin] and incubated for 30 min on ice followed by vigorous voltex for 10 sec. The lysates were centrifuged, and the supernatants containing the nuclear proteins were transferred to a new vial.

Western blot analysis

The induction of NF-κB protein was determined by Bio-Rad protein assay (Bio-Rad Labs inc., Hercules, CA, USA). The samples, 30 ㎍ of total protein per lane, were subjected to electrophoresis under a reducing condition on a 12 % SDS-PAGE gel and then transferred onto a nitrocellulose membrane at constant current of 100 V for 1.5 h in Transfer buffer (20 mM Glycine, 15.6 mM Tris-Base and 20 % Methanol). The antibodies for NF-κB were obtained from Santa Cruz Biotech (Santa Cruz Biotech, inc., Santa Cruz, CA, USA). All of antibodies were diluted in 5 % non-fat milk. After staining with the primary antibody, the membrane was subsequently incubated with the biotinylated secondary antibody for 1 h. After extensive washing with PBS, the membrane was coated with ECL (Amersham biosciences, Amersham, UK) for 1 min and the signal captured with X-ray film. The amount of expressed protein was determined using a scanner TouchToss SIS-3800 (Samsung, Seoul, Korea).

RESULTS

1. NF-κB activation induced by *A. actinomycetemcomitans* LPS

In order to investigate the signal transduction pathway induced by *A. actinomycetemcomitans* LPS at the level of gene expression, microarray was carried out using a GEarray method. Gene array membrane (GEarray Signal Transduction Pathway Finder) contains marker genes associated with 18 signal transduction pathways (Mitogenic Pathway, Wnt Pathway, Hedgehog Pathway, TGF- β Pathway, Survival Pathway, p53 Pathway, Stress Pathway, NF-kB Pathway, NFAT Pathway, CREB Pathway Jak-Stat Pathway, Estrogen Pathway, Androgen Pathway, Calcium and Protein Kinase C Pathway, Phospholipase C Pathway, Insulin Pathway, LDL Pathway and Retinoic Acid Pathway) and four housekeeping genes including glyceraldehydes-3-phosphate dehydrogenase (GAPDH), cyclophilin A, ribosomal protein L13a, and β-actin.

Figure 2 shown the results of microarray spots induced by *A.actinomycetemcomitans* LPS. When RAW264.7 cells were treated with *A. actinomycetemcomitans* LPS for 2 h and 18 h respectively, the spots were

shown up more than the control. *A. actinomycetemcomitans* LPS induced gene expression profiles in signal transduction pathway were summarized in Table 1. *A. actinomycetemcomitans* LPS was increased mRNA levels of TGF-β Pathway gene (Cdkn1a), p53 Pathway gene (Cdkn1a), NF-κB pathway genes (Nfkb1, Nfkbia, Icam1, Tnf), Estrogen pathway gene (Cdkn1a), Phospholipase C pathway genes (Junb, Icam1, Ptgs2), and LDL pathway genes (Csf2, Ccl2). Figure 3 shown the results of signal transduction pathway associated genes which are expressed by *A. actinomycetemcomitans* LPS. The results shown that NF-κB pathway was remarkably expressed by *A. actinomycetemcomitans* LPS. Depression profiles of signal transduction pathway finder genes were summarized in Table 2. *A. actinomycetemcomitans* LPS decreased mRNA levels of Wnt pathway gene (Ccnd1), P13kinase/AKP pathway gene (Ccnd1), p53 pathway gene (Bax), Stress pathway genes (Hsf1, HSP90-Rik), Estrogen pathway gene (Cdk2) and Insulin pathway gene (Fasn). The results of signal transduction pathway associated genes, which depressed by *A. actinomycetemcomitans* LPS, were shown in Figure 4.

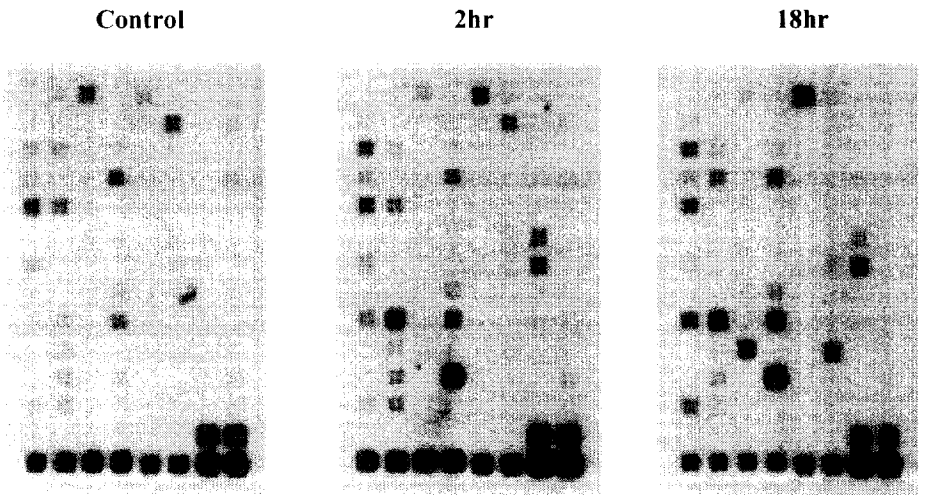

Figure 2. Microarray spots induced by *A. actinomycetemcomitans* LPS in RAW264.7 cells.

cDNA microarray analyses genes induced by *A. actinomycetemcomitans* LPS in RAW264.7 cells using the signal transduction pathway finder.

Table 1. Genes expressed in RAW264.7 cells induced by *A. actinomycetemcomitans* LPS

Pathway	Symbol	Gene Name	Description	2hr	18hr
Mitogenic Pathway	Jun	c-Jun	Jun oncogene	1.1	2.7
Wnt Pathway	Jun	c-Jun	Jun oncogene	1.1	2.7
TGF-B Pathway	Cdkn1a	p21Waf1/p21cip	Cyclin-dependent kinase inhibitor 1A(p21)	4.28	4.61
P13kinase/AKP Pathway	Jun	c-Jun	Jun oncogene	1.1	2.7
Jak/Src Pathway	Bcl2l1	Bcl-x	Bcl-like1	1.07	1.43
p53 Pathway	Cdkn1a	p21Waf1/p21cip	Cyclin-dependent kinase inhibitor 1A(p21)	4.28	4.61
	Mdm2	Mdm2	Transformed mouse 3T3 cell double minute2	1.45	1.66
NF-κB Pathway	Bcl2a1d	Bfl-1	B-cell leukemia/lymphoma 2 related protein A1d	4.44	5.69
	Nfkb1	NfkB1	Nuclear factor of kappa light chain gene enhancer in B-cells 1, p105	3.37	4.53
	Nfkbia	ikBa/Mad3	Nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, alpha	10.89	11.33
	Icam1	ICAM-1	intercellular adhesion molecule	8.86	6.43
	Tnf	TNFa	Tumor necrosis factor	14.14	14.71

Table 1. Continued

Pathway	Symbol	Gene Name	Description	2hr	18hr
Jak-Stat Pathway	Irf1	IRF-1	interferon regulatory factor 1	1.2	2.2
Estrogen Pathway	Cdkn1a	p21Waf1/p21cip	Cyclin-dependent kinase inhibitor 1A(p21)	4.28	4.61
Calcium and protein Kinase C Pathway	Csf2	GM-CSF	Colony stimulation factor 2(granulocyte-macrophage)	0.83	4.06
	Jun	c-Jun	Jun oncogene	1.1	2.7
	Odc	Ornithine decarboxylase	Ornithine decarboxylase, structural	2.49	2.86
Phospholipase C Pathway	Jun	c-Jun	Jun oncogene	1.1	2.7
	Junb	Jun-B	Jun-B Oncogene	10.29	11.57
	Icam1	ICAM-1	intercellular adhesion molecule	8.86	6.43
	Ptgs2	Cox-2	Prostaglandin-endoperoxide synthase 2	3.11	11
LDL Pathway	Csf2	GM-CSF	Colony stimulation factor 2(granulocyte-macrophage)	0.83	4.06
	Ccl2	Scya2	Chemokine(C-C motif) ligand 2	4	22
Insulin Pathway	Cebpb	C/EBP beta	CCAAT/enhancer binding protein(C/EBP), beta	1.28	1.16
Retinoic Acid Pathway	Ctsd	Cathepsin D	Cathepsin D	0.78	1
	Stra6	Stra6	Stimulated by retinoic acid gene 6	1.61	1.26

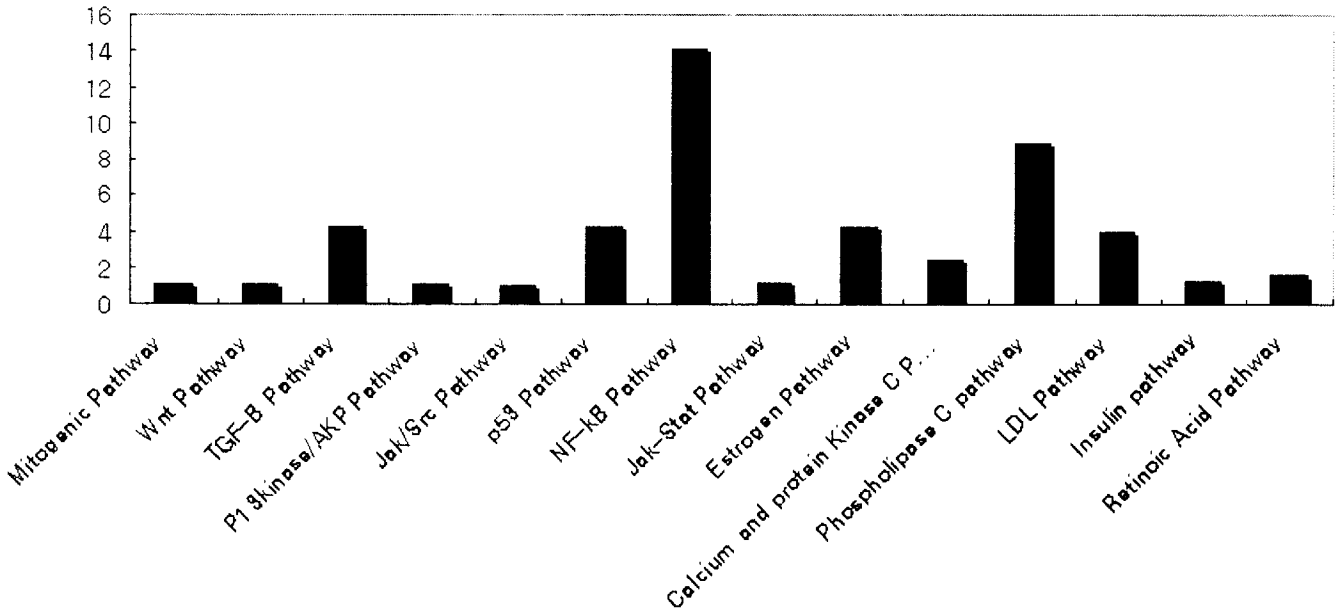

Figure 3. Comparison of signal transduction pathway expressed by *A. actinomycetemcomitans* LPS

Table 2. Genes depressed in RAW264.7 cells induced by *A. actinomycetemcomitans* LPS

Pathway	Symbol	Gene Name	Description	2hr	18hr
Wnt Pathway	Ccnd1	Cyclin D1	Cyclin D1	0.84	0.09
P13kinase/AKP	Ccnd1	Cyclin D1	Cyclin D1	0.84	0.09
Pathway	Pten	PTEN	Phosphatase and tensin homolog	1.54	0.75
p53 Pathway	Bax	Bax	Bcl2-associated X protein	0.52	0.31
	Hsf1	Hsf1(tcf5)	Heat shock factor 1	0.6	0.28
Stress Pathway	HSP90-Rik	HSP90/CDw52	RIKEN cDNA 2410016O06 gene, human HSP90 homolog	0.77	0.31
	Trp53	p53	Transformation related protein 53	1.94	0.75
NF-kB Pathway	Il2	IL-2	interleukin 2	1.27	0.65
NFAT Pathway	Il2	IL-2	interleukin 2	1.27	0.65
Estrogen Pathway	Cdk2	Cdk 2L	Cyclin dependent kinase 2	0.75	0.2
Calcium and protein	Il2	IL-2	interleukin 2	1.27	0.65
Kinase C Pathway	Tfrc	Tfrc/Trfr/Tfr1	Transferrin receptor	2.91	1
Insulin pathway	Fasn	fatty acid synthase	fatty acid synthase	0.92	0.11

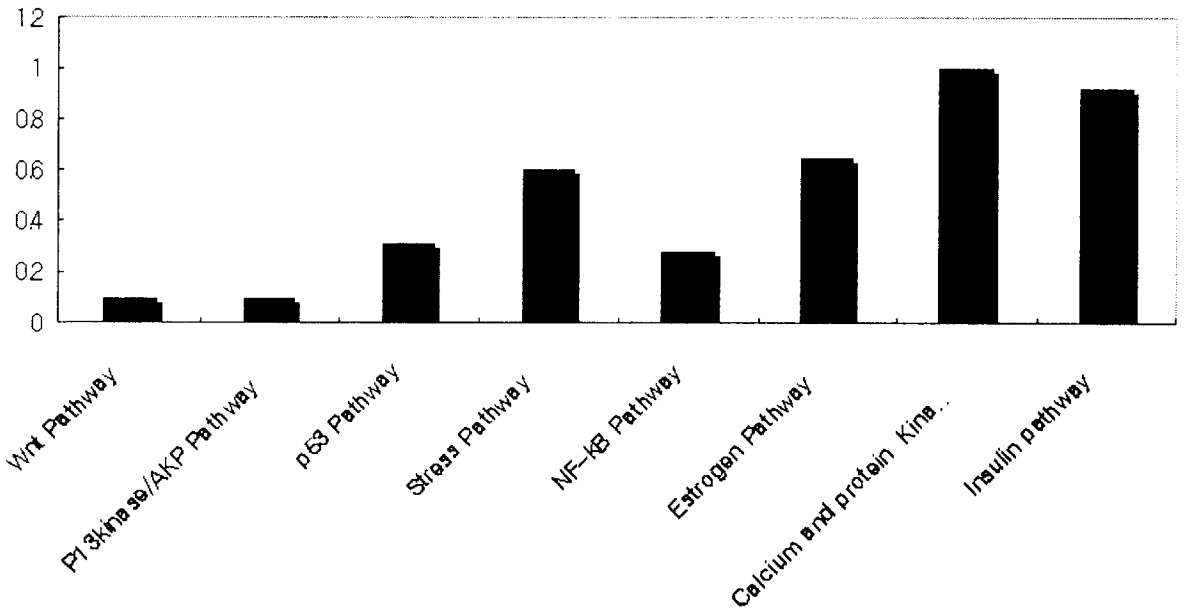

Figure 4. Comparison of signal transduction pathway depressed by *A. actinomycetemcomitans* LPS

2. *A. actinomycetemcomitans* LPS induced TNF- α and IL-1β expression through NF-κB pathway.

2-1. *A. actinomycetemcomitans* LPS induces TNF-α and IL-1β gene expression and protein synthesis.

To clarify whether *A. actinomycetemcomitans* LPS can induce TNF-α and IL-1β gene expression in mouse macrophage cell line, RAW 264.7 cells were treated with 100 ng/ml of *A. actinomycetemcomitans* LPS. Total RNA was prepared at 30min, 1h, 2h, 4h, 8h, 12h, 18h, and 24h after *A. actinomycetemcomitans* LPS treatment, respectively. The expression levels of TNF-α and IL-1β mRNA were measured by RT-PCR and shown in Figure 5. After treatment with *A. actinomycetemcomitans* LPS, TNF-α mRNA was showed two expression peaks at 2 h and 18 h and IL-1β mRNA was continually increased from 1 h. The expression of β-actin was used as a control gene. This experiment indicates that *A. actinomycetemcomitans* LPS induced TNF-α and IL-1β mRNA expression.

When RAW 264.7 cells were treated with 100 ng/ml of *A. actinomycetemcomitans* LPS for 30min, 1h, 2h, 4h, 8h, 12h and 24h, the production levels of TNF-α and IL-1β protein in culture supernatants were

measured by ELISA and shown in Figure 6 and 7. The production of TNF-α protein was detected at 1h and reached to the maximum after treatment at 2 h. The production of IL-1β protein was detected at 4 h and increased in a time-dependent manner. In these experiments, I confirmed that *A. actinomycetemcomitans* LPS leads to the induction of TNF-α and IL-1β protein production.

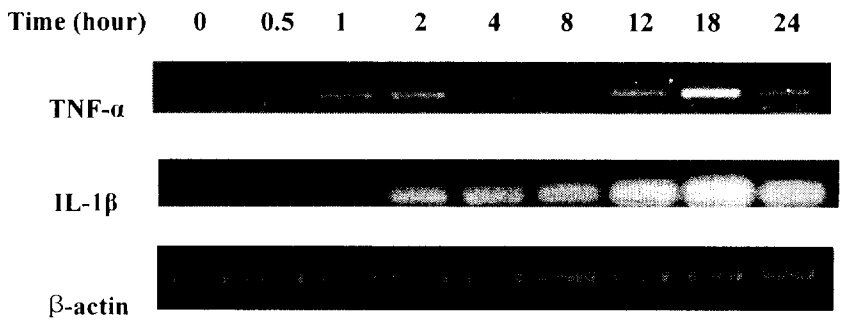

Figure 5. Expression levels of TNF-α and IL-1β mRNA induced by *A. actinomycetemcomitans* LPS

RAW 264.7 cells were treated with 100 ng/ml of *A. actinomycetemcomitans* LPS and total RNA was prepared at 30 min, 1 h, 2 h, 4 h, 8 h, 12 h, 18 h and 24 h after *A. actinomycetemcomitans* LPS treatment. The expression levels of TNF-α and IL-1β mRNA were measured by RT-PCR. The expression of β-actin gene was used as a control gene. RT-PCR was performed as described in the Materials and Methods.

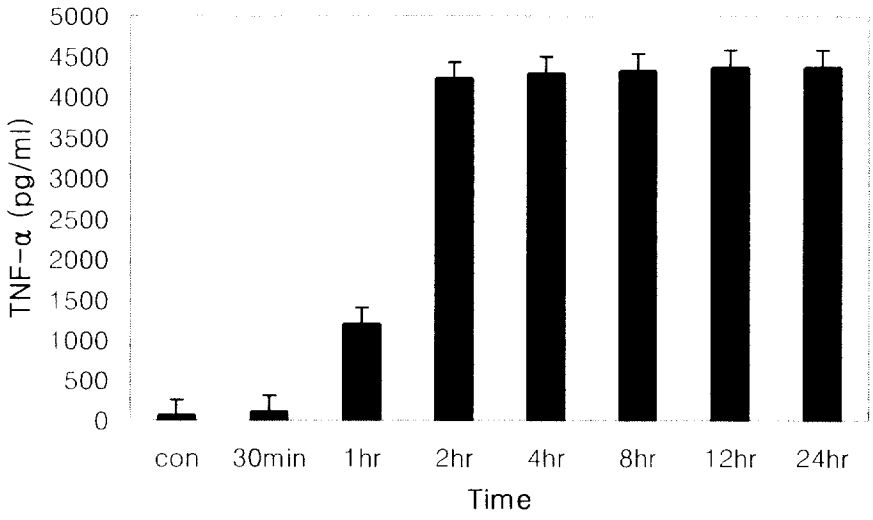

Figure 6. Production of TNF-α protein after treatment with *A. actinomycetemcomitans* LPS

RAW 264.7 cells were treated with 100 ng/ml of *A. actinomycetemcomitans* LPS and culture supernatants were prepared at 30 min, 1 h, 2 h, 4 h, 8 h, 12 h and 24 h after *A.actinomycetemcomitans* LPS treatment. The levels of TNF-α protein production in culture supernatants were measured by ELISA. Values are expressed as mean ± SD obtained from three independent experiments.

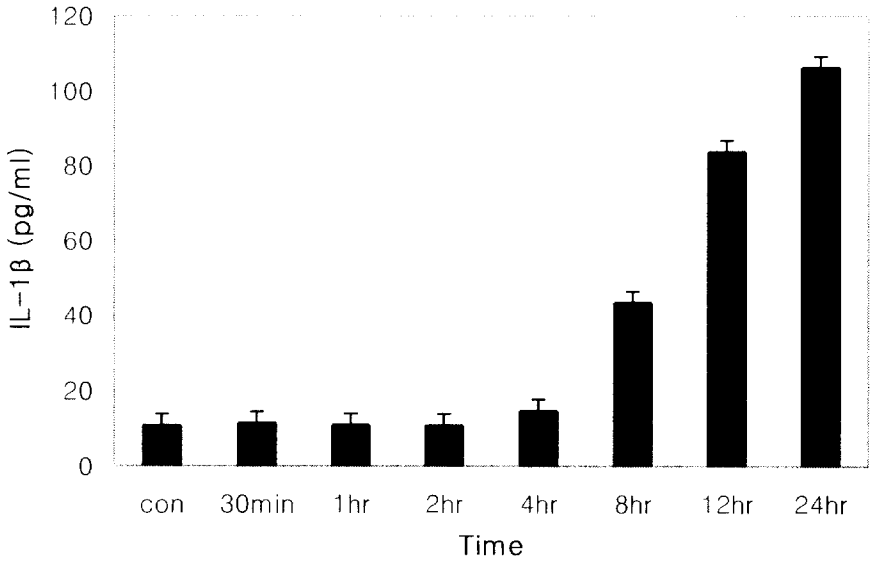

Figure 7. Production of IL-1β protein after treatment with *A. actinomycetemcomitans* LPS

RAW 264.7 cells were treated with 100 ng/ml of *A. actinomycetemcomitans* LPS and culture supernatants were prepared at 30 min, 1 h, 2 h, 4 h, 8 h, 18 h and 24 h after *A. actinomycetemcomitans* LPS treatment. The levels of IL-1β protein production in culture supernatants were measured by ELISA. Values are expressed as mean ± SD obtained from three independent experiments.

2-2. NF-κB activation induced by *A. actinomycetemcomitans* LPS

To elucidate whether NF-κB is involved in *A. actinomycetemcomitans* LPS-induced TNF-α and IL-1β expression, RAW 264.7 cells were treated with 100 ng/ml of *A. actinomycetemcomitans* LPS. Nuclear extracts was prepared at 10 min, 20 min, 30 min, 1 h, 2 h, 4 h and 8 h after *A. actinomycetemcomitans* LPS treatments. The induction of NF-κB protein in nucleus was analyzed by Western blot and shown in Figure 8. The NF-kB protein was strongly detected with a protein band of 65 kDa at 20 and 30min after treatment with *A. actinomycetemcomitans* LPS. The expression of β-actin protein was wed as a control. These data suggest that the production of TNF-α and IL-1β protein induced by *A. actinomycetemcomitans* LPS may be regulated by NF-κB at the transcriptional level.

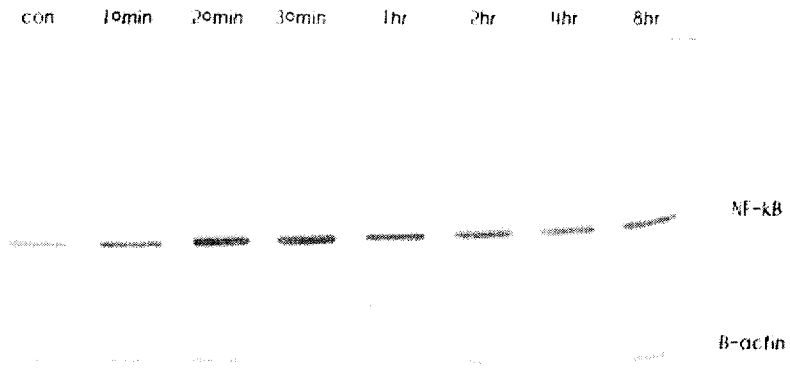

Figure 8. Western Blot analysis of NF-kB activation induced by *A. actinomycetemcomitans* LPS

RAW 264.7 cells were treated with 100 ng/ml of *A. actinomycetemcomitans* LPS and nuclear extracts was prepared at 10 min, 20 min, 30 min, 1 h, 2 h, 4 h and 8 h after *A. actinomycetemcomitans* LPS treatments. The induction of NF-κB protein in nucleus was analyzed by Western blot. The expression of β-actin protein was used as a control.

2-3. Pyrrolidine dithiocarbamate inhibited both the gene expression and protein synthesis of TNF-α and IL-1β induced by *A. actinomycetemcomitans* LPS.

To elucidate whether NF-κB is involued in *A. actinomycetemcomitans* LPS-induced TNF-α and IL-1β expression, RAW 264.7 cells were pretreated with 50 μM pyrrolidine dithiocarbamate (PDTC), NF-κB inhibitor, for 30 min and then treated with 100 ng/ml of *A. actinomycetemcomitans* LPS for 2 hr. The expression patterns of TNF-α and IL-1β mRNA were examined by RT-PCR and shown in Figure 9. After pretreatment with PDTC for 30 min, the expression of TNF-α (Figure 9A) and IL-1β (Figure 9B) mRNA induced by *A. actinomycetemcomitans* LPS were completely inhibited. These data indicated that the expression of TNF-α and IL-1β mRNA induced by *A. actinomycetemcomitans* LPS was related to NF-κB activation.

When RAW 264.7 cells were treated with *A. actinomycetemcomitnas* LPS for 2 h, 12 h, and 24 h after pretreatment with 50 μM PDTC for 30 min, the production levels of TNF-α and IL-1β protein in culture supernatnats were measured by ELISA and shown in Figure 10 and 11. When RAW 264.7 cells was only treated by *A. actinomycetemcomitans* LPS, TNF-α protein was produced 4067 pg/ml at 2 h and 4024 pg/ml at 12 h. After pretreatment with

PDTC, TNF-α protein was produced 624 pg/ml at 2 h and 2035 pg/ml 12 h. In the results, the production of TNF-α protein was inhibited 85 % at 2 h and 52 % at 12 h after pretreatment with PDTC. Also, RAW 264.7 cells was only treated by *A. actinomycetemcomitans* LPS, IL-1β protein was produced 8 pg/ml at 8 h, 18 pg/ml 12 h, and 32 pg/ml at 24 h. After pretreatment with PDTC, IL-1β protein produced 0.7 pg/ml at 8 h, 0.8 pg/ml at 12 h, and 1.1 pg/ml at 12 h. The production of IL-1β protein was completely inhibited until 24 h after pretreatment with PDTC. These data indicate that the production of TNF-α and IL-1β protein induced by *A. actinomycetemcomitans* LPS was related to NF-κB activation. These date confirm that *A. actinomycetemcomitans* LPS induced TNF-α and IL-1β genes through NF-κB pathway.

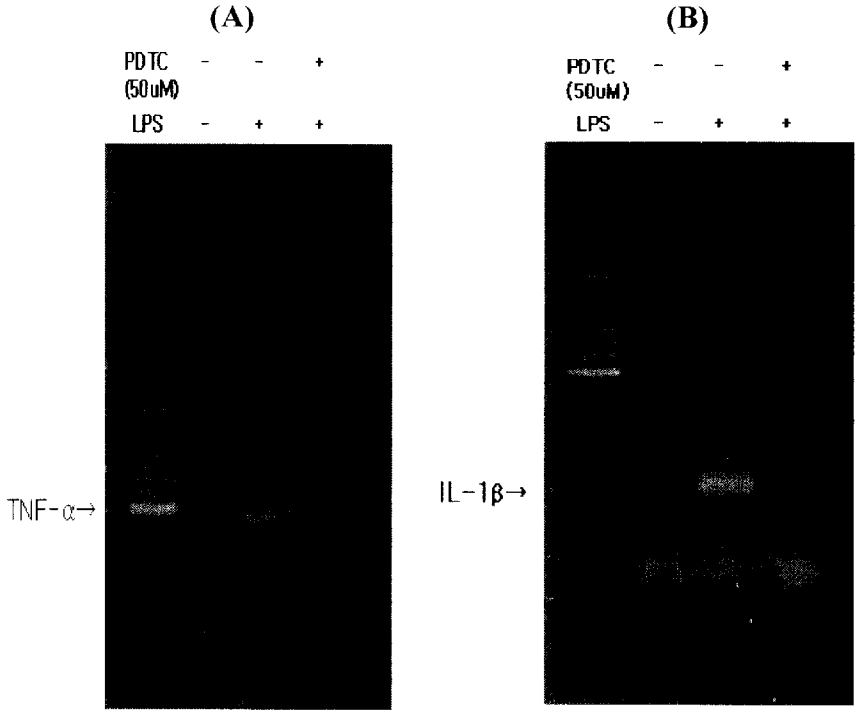

Figure 9. Expression pattern of TNF-α (A) and IL-1β (B) mRNA after treatment with PDTC.

RAW 264.7 cells were pretreated with 50 μM PDTC, NF-κB inhibitor, for 30 min and then treated with 100 ng/ml of *A. actinomycetemcomitans* LPS for 2 h. The expression patterns of TNF-α and IL-1β mRNA were examined by RT-PCR. RT-PCR was performed as described in the Materials and Methods.

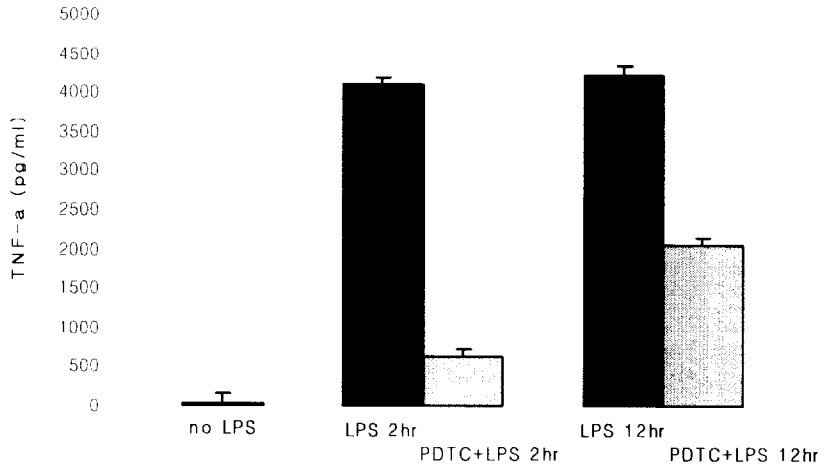

Figure 10. Production of TNF- α protein after treatment with PDTC.

RAW 264.7 cells were pretreated with 50 μM PDTC, NF-κB inhibitor, for 30 min and then treated with 100 ng/ml of *A. actinomycetemcomitans* LPS for 2 h and 12 h. The levels of TNF-α protein production in culture supernatants were measured by ELISA. Values are expressed as mean ± SD obtained from three independent experiments.

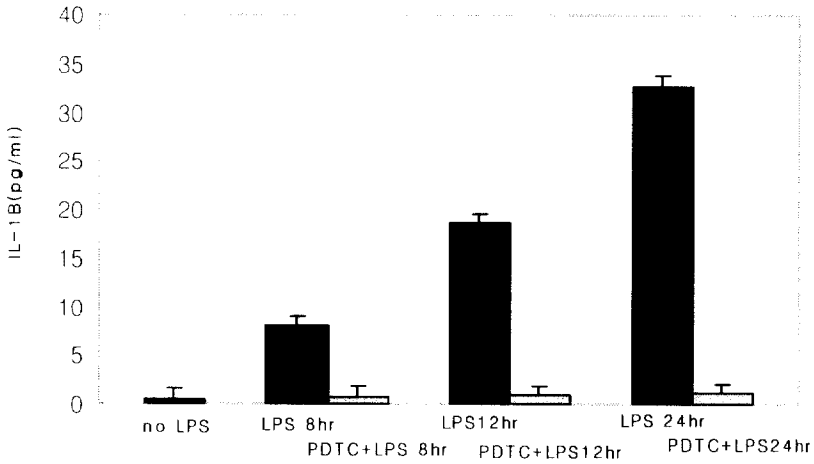

Figure 11. Production of IL-1 β protein after treatment with PDTC.

RAW 264.7 cells were pretreated with 50 μM PDTC, NF-κB inhibitor, for 30 min and then treated with 100 ng/ml of *A. actinomycetemcomitans* LPS for 2 h, 12 h, and 24 h. The levels of TNF-α protein production in culture supernatants were measured by ELISA. Values are expressed as mean ± SD obtained from three independent experiments.

DISCUSSION

Periodintal disease in its widest sense includes all disorders of the supporting structures of the teeth, namely the gingiva, periodontal ligament, and supporting alveolar bone. This may vary from inflammation of the gingiva alone, termed gingivitis, to the severe inflammation of the periodontal ligament called periodontitis, in which there is destruction of alveolar bone and eventual tooth loss.

In periodontitis, there is a progressive change in the composition of the microflora from aerobic, non-motile, Grampositive cocci to anaerobic, motile, Gram-negative bacilli. Some Gram-negative bacteria implicated in the aetiology of periodontal disease include *Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia, Bacteroides forsythus, Fusobacterium nucleatum, and Capnocytophaga* Spp. *Actinobacillus actinomycetemcomitans* is a major pathogenic bacterium and is associated with aggressive periodontal disease, which can lead to a rapid loss of alveolar bone and teeth. Thus, *A. actinomycetemcomitans* LPS is associated with the development and progression of periodontal disease. LPS also activate cells related to the innate immune system and thus contribute to

inflammatory processes. However, there is very little information about the *A. actinomycetemcomitans* LPS-induced signal transduction pathway.

In this study, I confirmed that the signal transduction pathway or genes induced by *A.actinomycetemcomitans* LPS were examined using a microarray technique in murine macrophage cells.

The advent of DNA microarray technology has provided a new and powerful tool that allows the simultaneous analysis of a large number of nucleic acid hybridizations in a rapid and efficient fashion. This technology is extremely useful in understanding the interrelationship of gene expression involved in the immune systems.

As a results of microarray analysis using Signal Transduction PathwayFinder GEarray, the expression of Cdkn1a, Nfkb1, Nfkbia, Icam1, tnf, Junb, Ptgs2, Ccl2 was up-regulated by A. *actinomycetemcomitans* LPS at transcription level in RAW 264.7 cells (Table 1). On the other hand, the expression of Ccnd1, Bax, Hsf1, Cdk2, and Fasn was down-regulated (Table 2). Some of these genes, gene expression of Nfkb1, Nfkbia, Icam1, and tnf which is related NF-κB pathway was remarkably up-regulated by A. *actinomycetemcomitans* LPS (Figure 3). Thus, these data suggest that *A. actinomycetemcomitans* LPS may induced NF-κB activation at transcription level. NF-κB is a multi-unit transcription factor, which plays a central role in

induction of pro-inflammatory cytokines and other many immunoregulatory genes.

Many studies have shown that the bacterial component of *A. actinomycetemcomitans* are able to induce production of inflammatory cytokines, IL-6, IL-1, TNF-α, MIP-1, MCP-1, and other, by several kinds of cells. Some of cytokines, IL-1β and TNF-α play important role in aggressive inflammatory processes, including the initiation and exacerbation of periodontal diseases, by promoting osteoclast formation and alveolar bone resorption. TNF-α and IL-1β stimulates the proliferation of many cell types through NF-κB activation.

The present study showed that *A. actinomycetemcomitans* LPS led to induce TNF-α and IL-1β mRNA expression and protein production in mouse macrophage cell line, RAW 264.7 cells. Also, *A. actinomycetemcomitans* LPS was induced NF-κB activation in nucleus. As shown in Figure 8, the induction of NF-κB was identified at 20 min after *A. actinomycetemcomitans* LPS treatment. As shown in Figure 5, 6, and 7, TNF-α and IL-1β mRNA expression was detected at 30 min, the protein was produced at 1 h after *A. actinomycetemcomitans* LPS treatment. As the results, I assumed that *A. actinomycetemcomitans* LPS was induced the inflammatory cytokines through NF-kB activation.

Thus, I investigated whether NF-kB is involved in *A. actinomycetemcomitans* LPS-induced TNF-α and IL-1β expression and protein production. When RAW 264.7 cells were treated with *A. actinomycetemcomitans* LPS after pretreatment with PDTC, NF-κB inhibitor, it leads to the inhibition of TNF- and IL-1β mRNA expression and protein production (Figure 7, 8, 9 and 10). These results indicate that *A. actinomycetemcomitans* LPS is a potent inducer of NF-κB and induces TNF-α and IL-1β gene expression through the activation of NF-κB.

Understanding the signal transduction pathways and the functions of those genes involved in immune response induced by *A. actinomycetemcomitans* LPS may facilitate the improvement therapy or vaccines for periodontal diseases caused by *A. actinomycetemcomitans*.

국문초록

치주염은 치아를 지지하고 있는 조직에 염증반응이 일어나는 질병으로 그람음성균의 감염으로 유발되는 질병이다. 급성 치주염의 주요한 발병인자로 알려진 *Actinobacillus actinomycetemcomitans* 의 Lipopolysaccharide(LPS)는 주요 독성인자로써 이에 대해 유도되는 signaling 에 대해서는 아직 잘 알려져 있지 않다.

본 연구에서는 DNA microarray 기법을 이용하여 *A. actinomycetemcomitans* LPS 에 의해 자극된 Murine macrophage cell 인 RAW 264.7 에서 발현되는 유전자를 확인하였다. 본 논문에서는 *A.actinomycetemcomitans* LPS 에 의해 특이적으로 활성화되는 신호전달계 또는 유전자의 발현을 확인하기 위해 "signal transduction pathway finder gene expression profiles"을 분석하였는데 특히 NF-κB pathway 에 관련된 유전자의 발현이 두드러지게 발견되었다.

실제로 세포 내에서 NF-κB pathway 에 관련된 유전자의 발현이 *A.actinomycetemcomitans* LPS 에 의해 유도되는지를 확인하기 위해 pro-inflammatory cytokine 인 TNF-α 와 IL-1β 의 유전자 발현과 단백질합성을 RT-PCR 과 ELISA 로 확인한 결과 TNF-α 는 mRNA 발현과 protein 합성이 2 시간 이후에 가장 많이 나타났고 IL-

1β 는 mRNA 는 2 시간 이후 protein 은 4 시간이후 계속 증가하였다. Western blotting 결과 NF-κB protein 이 핵내에서 증가함을 확인하였고 NF-κB 억제제인 PDTC 를 처리하였을 때 TNF-α 와 IL-1β 의 mRNA 발현 및 단백질 합성이 감소되었다. 결론적으로 *A.actinomycetemcomitans* 의 LPS 는 mouse macrophage cell line 인 RAW 264.7 cell 에서 NF-κB 를 활성화 경로로 염증성 사이토카인인 TNF-α 와 IL-1β 를 활성화시킴으로서 급성치주질환의 진행에 영향을 주는 것으로 생각되어진다.

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