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Expression of Growth Factors and Secretory Leukocyte Protease Inhibitor (SLPI) in RAW264.7 Cells after Lipopolysaccharide (LPS) Stimulation

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대식세포주(RAW264.7)에서 박테리아성 지질다당류(LPS) 자극에 의한 분비백혈구단백분해효소억제제(SLPI)와 성장인자들의 발현

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ABSTRACT

Secretory leukocyte protease inhibitor (SLPI) was known as one of bacterial lipopolysaccharide (LPS)-induced products of macrophage. Macrophages play an important role in the development of inflammatory responses by secreting an array of cytokines and chemokines in a tissue microenvironment. To identify the function and relationship between potent growth factors and SLPI after LPS stimulation, we conducted reverse transcriptase polymerase chain reaction (RT-PCR) and Western blots for the detection of mRNA and protein expression of SLPI and growth factors such as VEGF, PDGF, bFGF after 100 ng LPS stimulation on the RAW264.7 cells. The result of RT-PCR was showed SLPI mRNA expression was increased from 60 min to 48 h in RAW 264.7 cells after incubation with LPS. VEGF and PDGF mRNA was expressed highly at initial stage by LPS stimulation.

The mRNA of bFGF and type I collagen was very weakly expressed after LPS stimulation. SLPI protein level was increased likely the mRNA levels in RAW 267.7 cells. Additionally, phase contrast and scanning electron microscopic observation demonstrated that the LPS induce the change of morphology of the RAW264.7 cells. From these results, it suggests that expression of SLPI by LPS treatment may associate with VEGF and PDGF expression in RAW264.7 cells.

Keywords : Growth factors, Lipopolysaccharide, RAW264.7, Secretory leukocyte protease inhibitor (SLPI)

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INTRODUCTION

Lipopolysaccharide (LPS) is one of the materials causing immune response. Secretory responses of the cytokine or the growth factors of macrophage to LPS can protect the host from infection but high levels, contribute to systemic inflammatory response syndrome and destruction of host itself (Jin et al., 1997). Many cells can produce the growth factors and cytokines in abnormal environment such as hypoxia and inflammation (Jeffrey et al., 1997). These factors are as known that the vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and platelet-derived growth factor (PDGF) (Leung et al., 1989; Sunderkotter et al., 1994; Slavin, 1995).

In the blood, LPS binds to its binding protein as known LPS-binding serum protein. In the cell surface of neutrophil, monocyte, and macrophage, LPS binds to CD14 and Toll-like receptor4 (TLR4) and then they secrete cytokines such as TNF-α, IL-1, IL-6, protease, and NO, which participate in inflammatory process. Excessive cytokines play a key role in generation of endotoxic shock cascade. And protease such as neutrophil elastase and catepsin G participate in endotoxin shock induced by LPS (Tkalcevic et al., 2000; Medzhitov, 2001; Underhill & Ozinsky, 2002). Also, concentration of LPS to induce inflammatory response indicates differences of expression among various factors (Botero et al., 2003).

Secretory leukocyte protease inhibitor (SLPI) is an 11.7-kDa cystein-rich protein, as the epithelial cell product found in saliva, seminal plasma, and cervical, nasal, and bronchial mucus. SLPI promotes wound healing and cell proliferation *in vitro*, inhibits HIV and bacterial infection (Laurie et al., 2002). In addition, it was recently reported that SLPI has causal role in malignant behavior of cancer cells (Nick et al., 2003). Also, it was LPS-induced product of macrophage and had the function that antagonizes their LPS-induced activation of pro-inflammation signaling factors (Fritz, 1988; Ohlsson et al., 1988; Abe et al., 1991).

The most of inflammation of gingival tissue is gradually processed by bacterial infection and excessive inflammatory responses disturb the regeneration of gingival tissue (Love & Jenkinson, 2002). Therefore it is essential that identify the molecules to reduce the inflammatory reaction within diseases including gingival inflammation. Also various concentration of LPS to the macrophages leads to the different expressions of inflammatory factors. Therefore optimal concentration of LPS to the macrophages is important factors which influenced expression of SLPI as well as that of growth factors. In this study, we have tried out to determine the expression and function of SLPI such as pro-cytokine with several growth factors (VEGF, bFGF, PDGF) and extra-cellular matrix by LPS stimulation in RAW264.7 cells which react sensitively during inflammatory response.

MATERIAL AND METHOD

1. Cell culture

The RAW264.7 cells were plated on 60 mm dishes (2×10^5 cells per dish) and cultured with Dulbecco's Modified Eagle's medium (DMEM; Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco), 100-units/mL penicillin (Gibco), and 100 g/mL streptomycin (Gibco), 0.25 g/mL amphotericin B as Fungizone (Gibco), 1X Non-Essential Amino Acids Solution (Gibco) in a humidified 5% CO_2 incubator at 37°C.

2. Reverse transcriptase polymerase chain reaction (RT-PCR) and Real-Time PCR

The RAW264.7 cells were exposed for 30 min, 60 min, 90 min, 24 h, and 48 h to 100 ng/mL *E. coli* k-235 strain LPS (Sigma Chemical Co., St. Louis, MO, USA).

Tri reagent (MRC Inc., Montgomery Rd, CIN, USA) was used according to the manufacture's instructions to extract total RNA. The Superscript one-step reverse transcriptase (Invitrogen, USA) was used for the cDNA synthesis. The Ex Taq polymerase (TaKaRa Korea, Seoul, KOR) was used for PCR amplification according to the manufacturer's instructions. First denaturation and final extension of all PCR products was archived by incubation for 5 min at 94°C and 5 min at 72°C, respectively and for Real-time PCR (fw: 5-GCCCGGGAAA-GCAGAGGTG-3; rev: 5-ACATTGGGAGGGTTAAG-CATCATACAT-3). Each annealing cycle was performed at 63°C (30 s) and 60°C (40 s). The forward, reverse primers and annealing temperatures were listed in Table 1.

3. Western blotting

To analysis of SLPI protein expression, we used the western blotting analysis. Proteins were extracted from cell lysates by using NP-40 lysis buffer containing 150 mM NaCl, 1% NP-40, 50 mM Tris-Cl (pH 7.4), 2 mM Na_3VO_4, 2 mM $Na_4P_2O_7$, 50 mM NaF, 2 mM EDTA (pH 7.4) and added a leupeptin, aprotinin as the protease inhibitor. In all experiments, equivalent amounts (30 μg) of protein were loaded on to SDS 15%-polyacrylamide gels. After transfer, the membrane was blocked with 5% non-fat dry milk and blotted with anti-rabbit SLPI antiserum (1 : 500), followed by goat anti-rabbit-IgG (1 : 10,000) and was developed by ECL (Amershamphamacia, UK).

4. Immunoprecipitation (IP)

Additionally, to analysis of secreted SLPI protein, the medium from cultured cells was used for immunoprecipitation. The protein G beads slurry was washed with PBS for three times and added the culture medium 500 μL to the protein G beads slurry. Anti-mouse SLPI antiserum, and immobilized protein G beads were incubated with the collected medium overnight at 4°C. After incubation, protein-bead-antibody complexes were washed with PBS and centrifuged at 10,000 ×g for 5 min. Immunoprecipitates boiled for 5 min in reducing SDS-PAGE sample buffer and loaded on to SDS/15%-polyacrylamide gels.

5. Microscopy and scanning electron microscopy

To observe the morphology of the RAW264.7 cells, the cells were treated with LPS (100 ng/mL) for 30 min, 60 min, 90 min, 24 h, and 48 h respectively. The image of the cells has taken by inverted microscope (Olympus, JPN). For the observation of fine surface morphology of the cells, scanning electron microscopic method was used. After treatment of LPS, the RAW264.7 cells were fixed in a solution containing 2.5% glutaraldehyde in phosphate-buffered saline (PBS, pH 7.4). OsO_4 was used for post fixation and then the samples were washed in PBS at three times and dehydrated by 70, 80, 90, 95% and, finally, 100% ethanol, respectively. Hexamethyldisilazane (HMDS, Sigma, USA) was used for drying and then the samples were observed using scanning electron microscope (Hitach, H4700, Japan) at 15 kV.

Table 1. The summary of PCR primer sequences, annealing temperatures and amplification cycles

Gene	5′ Forward	3′ Reverse	Temp (°C)	Cycles	Size
mSLPI	5′-cggaattccagagctcccctgccttc-3′	5′-gctctagacatagagaaatgaatgcgttt-3′	63	30	676 bp
mVEGF	5′-gtggacatcttccaggagta-3′	5′-atctgcaagtacgttcgttt-3′	60	35	382 bp
mPDGF-BB	5′-ctcttgactccaagaacctg-3′	5′-aatccatcagagaaggtgtg-3′	48	33	582 bp
mbFGF	5′-ggcttcttcctgcgcatcca-3′	5′-gctcttagcagacattggaaga-3′	53	35	354 bp
mCollagen type I	5′-attcggagctcaagatgtaa-3′	5′-cagtcaagtcctagccaaac-3′	49	33	191 bp
mGAPDH	5′-ccatggagaaggctggg-3′	5′-caaagatgtcatggatgacc-3′	55	35	199 bp

RESULTS

1. The mRNA expression of SLPI and growth factors in RAW264.7 cells by LPS treatment

As the result of RT-PCR experiment, expression of SLPI mRNA was gradually increased from 60 min (over the 1.6 folds) following the time course in RAW264.7 cells by LPS stimulation (100 ng/mL). And expression of VEGF mRNA was increased from 30 and 60 min (over the 1.7 folds). After then, it was decreased following the time course (Fig. 1A). Also expression of PDGF mRNA was increased at 30 min and 60 min (over the 1.4 folds). mRNA of bFGF was faintly expressed from 24 h and Collagen type I mRNA was very faintly expressed from 24 h to 48 h (Fig. 1A). The result of Real-Time PCR measuring determinate quantity of SLPI mRNA was increased from 30 min to 24 h but it was decreased from 48 h, which was equal the that of RT-PCR result (Fig. 1B). GAPDH which was regularly showed at the expression level, was used to PCR analysis as internal control group.

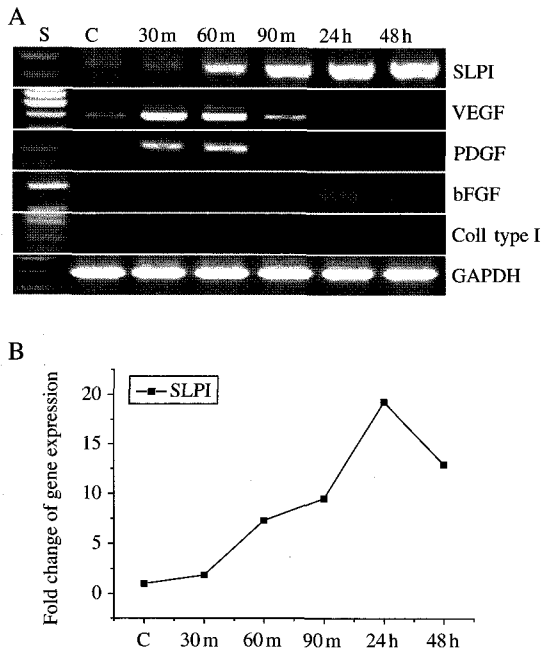

Fig. 1. RT-PCR assay of the SLPI and growth factors mRNA. A) SLPI and VEGF, PDGF, bFGF mRNA expression in RAW264.7 cells treated with LPS for control (lane 1, C), 30 min (lane 2), 60 min (lane 3), 90 min (lane 4), 24 hr (lane 5), and 48 hr (lane 6), respectively. GAPDH was used as an amplification internal control for the RT-PCR assays (S: 100 bp DNA size marker). B) Quantification of SLPI mRNA expression in RAW264.7 cells using Real Time-PCR method. SLPI mRNA expression in RAW264.7 cells treated with LPS for 30 min, 60 min, 90 min, 24 hr, and 48 hr, respectively. C, control.

2. SLPI protein expression in RAW264.7 cell lysate and supernatant

We performed the western blot analysis for determining the expression of SLPI protein levels in the cell lysates and culture medium of RAW 264.7 after LPS stimulation. It showed that the expression of SLPI was increased at 60 min and largely increased at 24 h (over

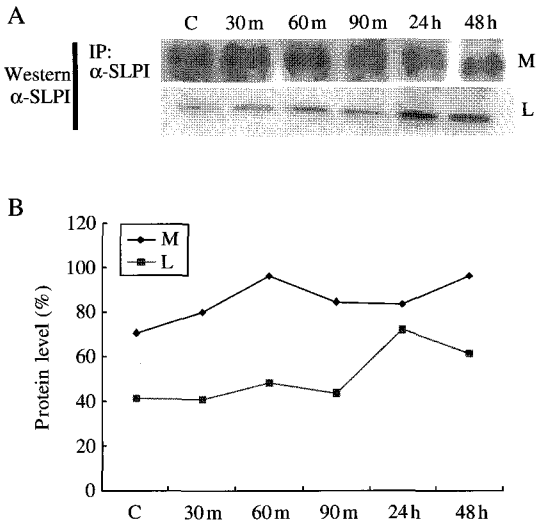

Fig. 2. SLPI protein levels in RAW264.7 cells by LPS treatment. A) To evaluatethe effect of LPS on SLPI protein expression in RAW264.7 cells (control, lane 1, C), we exposed to 100 ng/mL LPS for 30 min (lane 2), 60 min (lane 3), 90 min (lane 4), 24 hr (lane 5) and 48 hr (lane 6), collected the supernatant and performed immunoprecipitation. B) Percent of induction was quantified in the lysates and media by dividing the intensity of SLPI signals to that of control sample (M: medium, L: lysates). C, control.

1.7 folds) but it was decreased at 48 h compared with 24 h (Fig. 2A and 2B). Additionally, we have obtained a medium from cultured cells to analysis the expression of secreted SLPI protein. These results showed that amount of secreted SLPI protein was gradually increased until 60 min but it was gradually decreased from 90 min (Fig. 2A and 2B). These results were similarly to those of RT-PCR and Real-time PCR indicate that the induc-

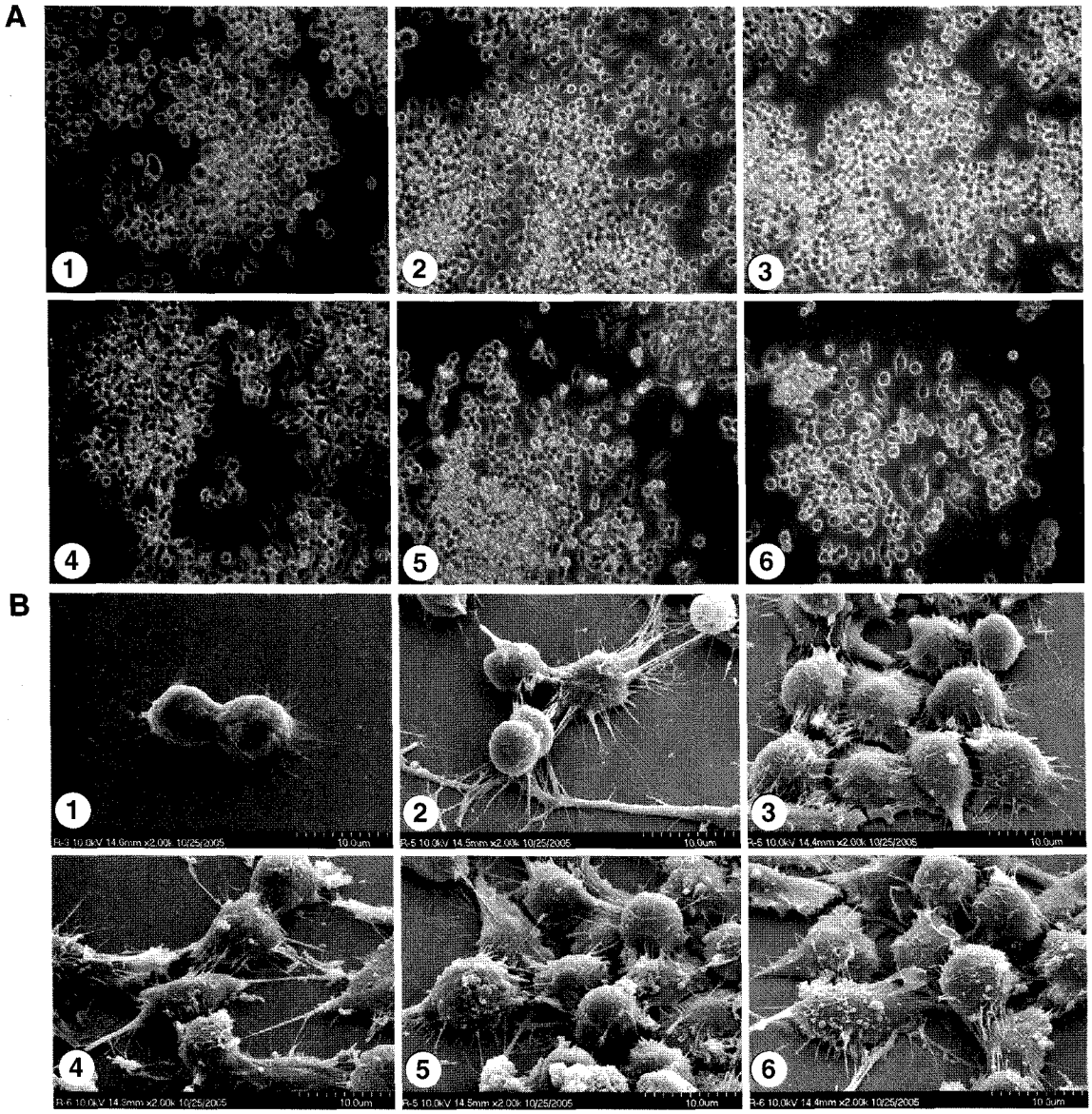

Fig. 3. Observation of morphological changes in RAW264.7 stimulation with LPS. A, Morphology of RAW264.7 cells observed using the phase contrast microscopy. The cells exposed to LPS (100 ng/mL) for control (①) and 30 min (②), 60 min (③) and 90 min (④), 24 hr (⑤), and 48 hr (⑥), respectively. All magnification is × 200. B, SEM analysis of RAW264.7 cells after stimulation with LPS. The change of morphology was observed from 30 (②) min after LPS treatmen. Control, (①); 60 min, (③); 90 min, (④); 24 hr, (⑤); 48 hr, (⑥).

tion of SLPI was gradually occurred by LPS signaling in cells.

3. Morphological changes in RAW264.7 cells after LPS treatment

By phase contrast microscopic analysis, we observed cell morphological changes after LPS stimulation. RAW264.7 cells showed spherical shapes at control group (untreated cells) (Fig. 3A-①) but spindle shapes was observed at 30 min after LPS stimulation (Fig. 3A-②). It was showed that spindle and long-stretched shapes at 60 min compared with 30 min (Fig. 3A-③). And it was observed more cells, which had fusiform, long-stretched vertical and horizontal, at 90 min by LPS stimulation (Fig. 3A-④). Also, the cells of groups for 24 h and 48 h had microspikes as compared with control group (Fig. 3A-⑤ and 3A-⑥). And the number of cells was increased by 60 min but it was gradually decreased at 90 min (Fig. 3A-③ and 3A-④).

We also observed microstructural changes of the RAW264.7 cells using SEM. The control cells showed spherical shapes with the same morphology observed by the phase contrast microscope and surface of the cells was smooth (Fig. 3B-①). In contrast, the morphology of cells after LPS stimulation showed the extension of the cytoplasm toward its outside at 30 min and had lots of cytoplasmic processes (Fig. 3B-②). The extension of cytoplasmic processes and number of microspikes were significantly increased from 60 min after stimulation of LPS (Fig. 3B-③). These similar morphological characteristics were observed from 90 min to 48 h (Fig. 3B-④, 3B⑤, and 3B⑥).

DISCUSSION

SLPI has been known as a potent inhibitor of leukocyte serine proteases, including elastase and cathepsin G from neutrophils, chymase and tryptase from mast cells, as well as trypsin and chymotrypsin from pancreatic acinar cells (Fink et al., 1986; Thompson & Ohlsson, 1986; Ohlsson et al., 1988). Also SLPI was expressed on inflammatory response cells such as spleen adhesion cell, peritoneal macrophage, bone marrow-derived macrophage and peritoneal polymorphonuclear leukocyte (Jin et al., 1997). Macrophage plays a key role in process of inflammatory response by secreting cytokines and chemokines in tissue. For example, pro-inflammatory cytokine such as IL-1 (interleukin-1) and TNF-α (Tumor necrosis factor-α) activate macrophage and increase the expression of other cytokines or chemokines (Matsuyama et al., 2004). The molecules related to inflammatory response were secreted to protect host tissues from infections. But in case of excessively secreted molecules, host was affected with these because of abnormal systemic inflammatory response (Bone, 1991; Marino et al., 1997). SLPI in activated RAW264.7 cells by LPS treatment increased expression of anti-inflammatory cytokine such as IL-10 (interleukin-10) and transforming growth factor-β (TGF-β) (Sano et al., 2000).

Macrophages participate in inflammatory processes and non-specific immunity by producing cytokines and mediators in response to LPS (Kaoru et al., 1997). It was known that SLPI was expressed and increased by LPS stimulation in this cell (Jin et al., 1998). Therefore, we observed expression of SLPI and several growth factors in RAW264.7 cells by LPS stimulation (100 ng/mL) as compared with previously reported results (50 mg/mL). The result of RT-PCR showed that mRNA of the SLPI was faintly expressed in LPS untreated control cells and mRNA of the SLPI was increased by LPS stimulation from 60 min. According to the previous results, SLPI was expressed from 90 min by LPS stimulation in lower concentration (1 ng and 10 ng) than 100 ng. Thus, it suggests that optimal concentration to expression of SLPI is to 100 ng as an inflammatory mediator. And SLPI induced the expression of genes which were related to cell growth and proliferation in endometrium epithelial cells (Zhang et al., 2002). In our results RT-PCR and Real-Time PCR showed that mRNA of SLPI

was faintly expressed in control cells by untreated LPS. Therefore, the expression of SLPI in control cells, it may act as the molecules for the cell growth and proliferation of the neighboring cells.

In this study, we performed the western blot analysis for determining the expression of SLPI protein levels in the lysates and culture medium of RAW 264.7 cells by LPS stimulation. Interestingly, intracellular SLPI protein was significantly increased from 24 h but secreted protein was increased at 48 h. In NIH3T3 cells, intracellular SLPI was increased at 24 h and 48 h after LPS stimulation and also secreted protein was increased at the same time (Lee et al., 2006). Therefore, it seems that the differential expression of intra and secreted SLPI protein is dependant on the cell types.

VEGF is one of the main control molecules participating in angiogenesis and it plays a key role in macrophage. Activated macrophage secretes several new angiogenesis factors in inflammatory responses (Folkman & Klagsbrun, 1987; Polverini, 1998). Also, expression of VEGF was increased by LPS and ovary steroid and it was expressed in peritoneal macrophage on endometriosis patients (McLaren et al., 1996). These results showed that activated macrophage participates partly in angionenesis and infiltration through secretion of VEGF (Perez-Ruiz et al., 1999). In dental pulp cells, the various concentrations of LPS do not affect the expression of VEGF mRNA and secreted VEGF protects dental pulp and other growth factor as well as condition of bacterial infection. mRNA of VEGF was consistently expressed in RAW264.7 cells by LPS concentration (20 mg) (Botero et al., 2003). This expression pattern was different from that of our results at the time of 30 min and 60 min. Therefore optimal concentration of LPS can affect to expression of SLPI as well as that of growth factors.

PDGF as representative mitotic growth factor was expressed various cells such as platelet, monocyte, ectodermal mesenchyme and vascular smooth muscle cell. Monocyte and macrophage play a key role in inflammatory response of atherosclerosis. Monocyte binds with suitable receptor in smooth muscle cell of blood vessel and then it secrets PDGF that differentiates monocyte to macrophage through a series of process (Reddy et al., 2003; Cai et al., 2004). PDGF was increased pocket epithelial tissue of gingival tissue in inflammatory response and it contributes to inflammatory response in periodontal disease process (Pinheiro et al., 2003). In this study, expression of PDGF was increased similar that of VEGF at early phase. Therefore, expression of PDGF may be related the differentiation of infiltrated monocyte through the blood vessel to macrophage during inflammatory response.

SPLI was increased by LPS stimulation from 60 min and it was expressed higher until 48 h but the expression of VEGF and PDGF was increased ahead of than 60 min and they were kept by 60 min. Then, it was significantly decreased. From our results of RT-PCR there was relation of SLPI, VEGF and PDGF. Even though SPLI controls inflammatory response in macrophage by autocrine (Jin et al., 1998), SLPI may be regulated by the expression of VEGF and PDGF after LPS stimulation but we need to perform additional experiments to observe the relationship of these factors by protein stimulation.

In vivo bFGF promotes neovascularization and formation of granulation tissue, production and secretion of bFGF in endothelial cell were promoted by pro-inflammatory cytokines such as IFN-α+ (interferone-α+), IL-2 (interleukin-2), IL-1β (interleukin-1β) and nitric oxide (NO) (Cozzolino et al., 1993; Walford & Loscalzo, 2003). Also, inflammatory response cells such as macrophage (Baird et al., 1985; Kuwabara et al., 1995), CD4+ and CD8+ T lymphocyte (Blotnick et al., 1994; Peoples et al., 1995) secrete bFGF. In this study, the result of RT-PCR showed that bFGF by LPS stimulation was faintly expressed in the second half of the stimulation. It was known that the expression of bFGF was not observed by LPS treatment but there was difference of expression amount according to bacterial amount and

infection time (Prochnau et al., 2004). From this, it assumes that bFGF may be induced by bacteria but not LPS in RAW264.7 cells. But the concentration of LPS will be important factor to express the bFGF.

Collagen is the most important component in extracellular matrix. Type I collagen is important structural constituent in extracellular matrix of the whole body and it was found in connective tissue, internal organic, skin, muscle, and bone. Macrophage secretes mainly collagenase by LPS or zymosan stimulation (Cury et al., 1988; Shapiro et al., 1992). Interestingly, mRNA of collagen was weekly expressed from 24 h and 48 h by LPS stimulation. From these results, collagen may participate in accommodation of extracellular matrix during remodeling of destructive tissue and inflammatory processes.

To identify the changes of cell morphology by optimal concentration of LPS (100 ng) in RAW 264.7 cell, we observed morphological changes using microscope. It showed that microspike, extension of cytoplasm and cytoplasmic processes by phase contrast microscope and scanning electron microscope. These characteristics are identical with previous results (Ross et al., 1995; Tian et al., 2000). However these studies were used high concentration of LPS (above 50 mg) at the stimulation, thus there were lots of microspikes and transformation of cell compared with 100 ng LPS treated groups. From these, it suggests that microspike and cytoplasmic processes are morphological characteristics during differentiation of monocyte after LPS stimulation in RAW264.7 cells.

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<국문초록>

분비백혈구단백분해효소억제제 (SLPI)와 여러 성장인자들은 상처 감염이나 박테리아 침입 시 일어나는 염증반응에 서로 관계가 있지만, 대식세포에서 LPS 자극시 SLPI와 VEGF, bFGF, PDGF 등과 같은 성장 인자들의 발현관계에 대해서는 아직까지 알려진 연구 결과가 없다. 따라서 본 연구는 대식세포 세포주로 알려진 RAW264.7 세포에 SLPI 발현의 적정농도인 LPS에 반응하는 SLPI 및 성장 인자들과 세포외부기질이 발현을 규명하고자 하였다.

역전사효소 중합반응 (RT-PCR)과 면역학적 단백질 검출법 (Western blotting)은 LPS 처리 후 SLPI와 몇몇 성장인자들 (VEGF, bFGF, PDGF)와 제1형 아교질 mRNA와 SLPI 단백질의 검출을 위해 수행하였다. RAW264.7 세포주를 mL 당 100 ng의 LPS에 각각 30분, 60분, 90분, 24시간, 48시간동안 노출시켰다.

RT-PCR 결과 SLPI mRNA는 시간이 지남에 따라 점점 발현 양이 증가하였고 VEGF와 PDGF mRNA는 초기에 높은 발현 양상을 보였다. 그러나 bFGF와 I형 아교질의 발현은 매우 미약하게 나타났다. SLPI 단백질 발현 역시 mRNA 수준과 마찬가지로 증가하는 양상을 보였는데, 배양액내의 SLPI 단백질양은 전체적으로 감소하는 경향을 보였다. 또한 광학현미경 관찰과 주사전자현미경 관찰 결과, LPS가 RAW264.7 세포주의 형태학적인 변화를 일으킴을 확인하였다.

본 결과를 종합하면 SLPI 발현증가의 적정 농도라 생각되는 100 ng의 LPS에 의해서 발현되는 VEGF나 PDGF는 SLPI의 발현에 관계가 있는 것으로 생각되지만 추후에 이들 인자들의 단백질이나 유전자 도입을 통하여 발현 관계를 명확히 확인해야 하는 추가실험이 진행되어야 할 것이다.