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Increased Protein of the Secretory Leukocyte Protease Inhibitor (SLPI) and the Expression of Growth Factors in NIH3T3 Cells by LPS Stimulation

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NIH3T3 세포주에서 LPS자극에 의한 분비백혈구단백분해효소억제제(SLPI)의 단백질증가와 성장인자들의 발현

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ABSTRACT

Secretory leukocyte protease inhibitor (SLPI) involves tissue protection against the destructive action of neutrophil elastase at the site of inflammation. Several studies on new functions of SLPI have demonstrated that SLPI may play a primary role in innate immunity than protease inhibitor. To identify the function of SLPI by lipopolysaccharide (LPS) stimulation in the embryonic fibroblast (NIH3T3) cells, we studied the expression of SLPI compared to other growth factors involving the LPS treatment. To address this, we performed the reverse transcriptase polymerase chain reaction (RT-PCR) and Western blots for the detection of mRNA and protein expression of the SLPI and some growth factors such as VEGF, bFGF, and PDGF-BB after LPS stimulation. NIH3T3 cells were exposed 100 ng/mL *Escherichia coli* LPS for 30 min, 60 min, 90 min, 24 h, and 48 h, respectively. The result of RT-PCR showed that SLPI and VEGF mRNA was expressed strongly in NIH3T3 without related to LPS stimulation. mRNA of bFGF was weakly expressed such as the expression of the control. PDGF mRNA expression gradually increased follows at time course. However, SLPI protein level was increased in lysates and culture medium by LPS stimulation. Phase contrast microscopic and scanning

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electron microscopic observation showed that the increased cell number and cytoplasmic enlargement of the NIH3T3 cells. Therefore, it suggests that the LPS upregulates SLPI expression in NIH3T3 cells. Moreover, secreted SLPI may stimulate cell proliferation and migration.

Key words : Growth factors, Lipopolysaccharide, NIH3T3, Secretory leukocyte protease inhibitor (SLPI)

INTRODUCTION

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 known LPS-induced product of macrophage and had the function that antagonizes their LPS-induced activation of pro-inflammation signaling factors (Fritz et al., 1988; Ohlsson et al., 1988, Abe et al., 1991). Besides, SLPI promotes wound healing and cell proliferation *in vitro*, inhibits HIV and bacterial infection (Laurie et al., 2002). In addition, SLPI was recently reported that causal role in malignant behavior of cancer cells (Nick et al., 2003).

The cell wall of Gram-negative bacteria contains lipopolysaccharide can stimulate the most LPS-responsive cells in the mammalian host. The macrophage secretory response 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). Fibroblasts are distributed in connective tissue of whole the body, where they secret a nonrigid extracellular matrix such as type I or type III collagen. When a tissue is injured by infection or wounding, the fibroblasts proliferate in adjacent of wound site and migrate into the wound. Fibroblasts produce various pro-cytokines, growth factors and large amounts of collagenous matrix for repairing of the damaged tissue or by LPS stimulation (Alberts et al., 2002; Kumagai et al., 2005). Many cells can produce the growth factors and cytokines in abnormal environment such as hypoxic and inflammatory, including tumor cells, keratinocytes, fibroblasts and macrophages (Jeffrey et al., 1997). These factors as known that the vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF) and so on (Leung et al., 1989; Sunderkotter et al., 1994; Slavin, 1995).

Inflammation of gingival tissue is mainly 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 reduced inflammatory duration in gingival inflammation. In this study, we have tried out determined expression and function of SLPI with several growth factors in NIH3T3 cells during inflammatory response.

MATERIAL AND METHOD

1. Cell culture

The NIH3T3 cells plated on 60 mm dishes (2×10^5 cells per dish). The cells were 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℃.

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

The NIH3T3 cells were exposed for 30 min, 60 min, 90 min, 24 h, 48 h to 100 ng/mL *E. coli* k-235 strain LPS (Sigma Chemical Co., St. Louis, MO, USA) and Tri

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

Gene	5′ Forward	3′ Reverse	Temp (°C)	Cycles	Size
mSLPI	5′-CGGAATTCCAGAGCTCCCCT-GCCTTC-3′	5′-GCTCTAGACATAGAGAAAT-GAATGCGTTT-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
mGAPDH	5′-CCATGGAGAAGGCTGGG-3′	5′-CAAAGATGTCATGGATGACC-3′	55	35	199 bp

reagent (MRC Inc., Cincinnati, OH, USA) was used according to the manufacture's instructions to extract total RNA. The Superscript one-step reverse transcriptase (Invitrogen, Carlsbad, CA, 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. The forward, reverse primers and annealing temperatures were listed in Table 1.

3. Antibody and Western blotting

The peptides EGGKNDAIKIGAC was selected among polypeptide region of the mouse SLPI (mSLPI) protein and it has generated through an order (Takara, Inc., KOR). To analysis of SLPI protein expression cytosolic form, 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 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 (Amersham Biosciences, Piscataway, NJ, USA).

4. Immunoprecipitation (IP)

Additionally, for immunoprecipitation, we have obtained a medium from cultured cells to analysis of SLPI protein expression secreted form. The protein G beads slurry (Kirkegaard & Perry Laboratories, Gaithersburg, ML, USA) washed in PBS for three times, added the culture medium 500 μL. 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 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. Observation of cell morphology

To observe the morphology of the NIH3T3 cells were exposed to LPS (100 ng/mL) for control, 30 min, and 90 min 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 NIH3T3 cells were fixed in a solution containing 2.5% glutaraldehyde in phosphate-buffered saline (PBS, pH 7.4). Post fixation was used by OsO_4 and then the samples were washed in PBS at three times and dehydrated by sequential washes in 70, 80, 90, 95% and, finally, 100% ethanol, respectively. Hexamethyldisilazane (Sigma Chemical Co) was used for drying and then the samples were observed using scanning electron microscope (Hitach, H4700, JPN) at 15 kv.

RESULT

1. The mRNA expression of SLPI and growth factors in NIH3T3 cells by LPS treatment

The SLPI and VEGF mRNA were strongly expressed without time course in NIH3T3 cells but expression of PDGF gradually increased follows the time course. In case of bFGF expression was decreased gradually follows the time course after LPS stimulation (Fig. 1). Constitutive expression of SLPI and VEGF indicates that SLPI including this growth factor was not affected by LPS treatment in NIH3T3 cells at mRNA levels. A GAPDH signal was indicated the same intensity for experimental confirming that equal amounts of templates.

2. Western blot analysis of SLPI protein expression in cell lysates and supernatant

We performed the western blot analysis for determining the expression of SLPI protein levels in the cell lysates and culture medium of NIH3T3 cells by LPS stimulation. Western blot analysis showed that the expression of SLPI protein was increased from 60 min (over the 0.5 fold) and then it was significantly expressed at 24 h and 48 h (over the 1.8 and 3.9 folds) in cell lysates.

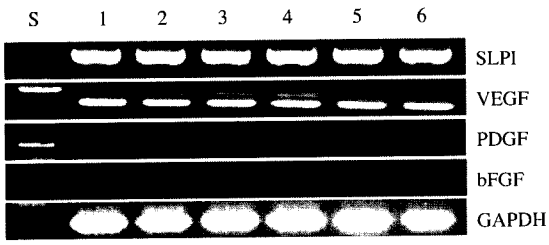

Fig. 1. RT-PCR assay of the SLPI and growth factors mRNA. SLPI and VEGF, PDGF, bFGF mRNA expression in NIH3T3 cells treated with LPS for control (lane 1), 30 min (lane 2), 60 min (lane 3), 90 min (lane 4), 24 h (lane 5), and 48 h (lane 6), respectively. GAPDH was used as an amplification internal control for the RT-PCR assays (S: 100 bp DNA size marker).

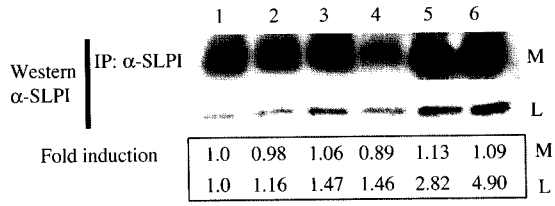

Fig. 2. SLPI protein levels in NIH3T3 cells by LPS treatment. To evaluate the effect of LPS on SLPI protein expression in NIH3T3 cells, we exposed to 100 ng/mL LPS for control (lane 1) 30 min (lane 2), 60 min (lane 3), 90 min (lane 4), 24 h (lane 5) and 48 h (lane 6), collected the supernatant and performed immunoprecipitation. Fold 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).

SLPI was constitutively secreted in culture media (Fig. 2). These results indicate that the induction of SLPI was gradually occurred by LPS signaling in NIH3T3 cells.

3. The observation of morphology in NIH3T3 after LPS treatment

Activated macrophage cells by LPS stimulation was known inducing the short cytoplasmic elongation of cells, but the gingival fibroblast did not show any detectable change by LPS stimulation compared with untreated cells (Botero et al., 2003). By phase contrast microscopic result, we also did not find any detectable change in morphology compared with untreated cells consistent with their results (Fig. 3A①-③) in NIH3T3 cells. However, the numbers of cells increased at 90 min in NIH3T3 cells by LPS stimulation (Fig. 3A③). Additionally, we added the SEM analysis to the NIH3T3 cells with LPS stimulation for the fine structural observation. The NIH3T3 cells without LPS treatment was showed the fusiform such as the common morphology of fibroblasts (Fig. 3B①). In contrast, the morphology of NIH3T3 cells by LPS stimulation was showed the extension of the cytoplasm at 30 and 90 min (Fig. 3B②, ③) compared with untreated cells. These results indicate that LPS induce the cytoplasmic extension in NIH3T3 cells.

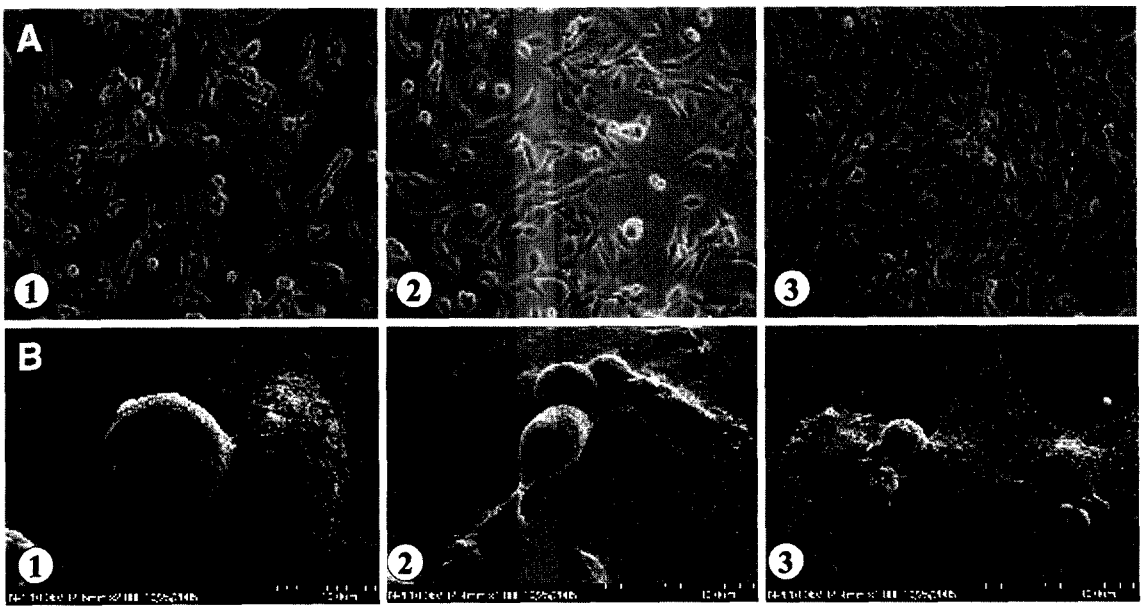

Fig. 3. Observation of morphology in NIH3T3 stimulation with LPS. A) Morphology of NIH3T3 cells observed using the phase contrast microscopy. The cells exposed to LPS (100 ng/mL) for control (①) and 30 min (②), and 90 min (③) respectively. All scale bars indicate 40 μm. B) SEM analysis of NIH3T3 cells stimulation with LPS. The change of morphology was represent at 60 min (③) by LPS compared with control (①) and 30 min (②) samples.

DISCUSSION

Fibroblasts participate in inflammatory processes and non-specific immunity by producing cytokines and mediators in response to LPS (Kaoru et al., 1997). Moreover, fibroblasts are activated more readily to produce nitric oxide than interstitial macrophages and may be the major source of this mediator in tissues (Lavnikova et al., 1995). Therefore, we prospect the induction of SLPI mRNA in NIH3T3 cells by LPS treatment. Unexpectedly, mRNA of the SLPI was strongly expressed in LPS untreated control cells and this expression was not affected by LPS all time course. In present study, mRNA expression of VEGF and bFGF was showed identical patterns with SLPI. The previous study showed the expression aspect of VEGF that regardless amount (0, 20 μg/mL) and time (0, 24 h) of the LPS presented constantly highly three cell types but VEGF was regulated primarily at the post-transcriptional level (Botero et al., 2003).

Therefore we conducted western blot analysis to detect the induction of SLPI at protein level. Interestingly, the SLPI was gradually increased at the protein level and also significantly expressed at the late time of the stimulation of LPS in concordance with VEGF results. The relatively high levels of VEGF secreted by unstimulated pulp cells provide a pro-angiogenic input that is necessary for the maintenance of pulp vascularization. This result indicates that VEGF involves not only pulp vascularization but also protect from bacterial challenges in odontoblast layers (Botero et al., 2003). SLPI has two distinct domains that one is anti-inflammatory and the other is anti-protease function (Thompson et al., 1986). SLPI was known that inhibits the several serine protease such as elastase, cathepsin G, trypsin, chymotrypsin, and chymase (Stetler et al., 1986). Therefore, the major physiological role of SLPI is considered to be

the protection of tissue from these proteases at site of inflammation. On the base of these previous reports, regulated transcription level of the SLPI in NIH3T3 cells indicates that the role of SLPI may be anti-protease function rather than that of anti-inflammatory by LPS stimulation.

It was showed that the PDGF is present in increased levels in the human inflamed gingiva and is mainly localized to the pocket epithelium. This demonstration indicates that chronic expression of PDGF contributes to the inflammatory changes that occur during periodontal diseases (Pinheiro et al., 2003). Additionally, it was reported that LPS up-regulates expression of PDGF-receptor alpha (Coin et al., 1996). Accordance with these reports, we showed that PDGF mRNA was gradually increased by LPS stimulation, suggesting that the NIH3T3 cells produce the PDGF in inflammatory response. In this study, bFGF was constitutively expressed and it seemed to be not effected by LPS stimulation. It was known that viable bacteria but not bacterial LPS may be essential for eliciting bFGF and it is expressed depending on the number of infecting bacteria and the time of infection as well (Prochnau et al., 2004). From this report, one possible explanation to the reason of the constitutively expression of bFGF mRNA was not to viral bacteria stimulation. Further we need to study whether induction of bFGF is occurred in protein level.

In our experiment, SEM analysis showed the cytoplasmic extension in NIH3T3 cells by LPS stimulation. The previous report, macrophages challenged with LPS presented short cytoplasmic elongations compared with untreated macrophages (Ross et al., 1995). Even though we do not explain the reason of the morphological change in NIH3T3 from our result, NIH3T3 cells were affected by LPS stimulation.

Fibroblast proliferation is one of the earliest features of fibrosis, preceding collagen deposition in wound. In this microscopic study, NIH3T3 cells seemed like enhanced proliferative activity by LPS stimulation. But

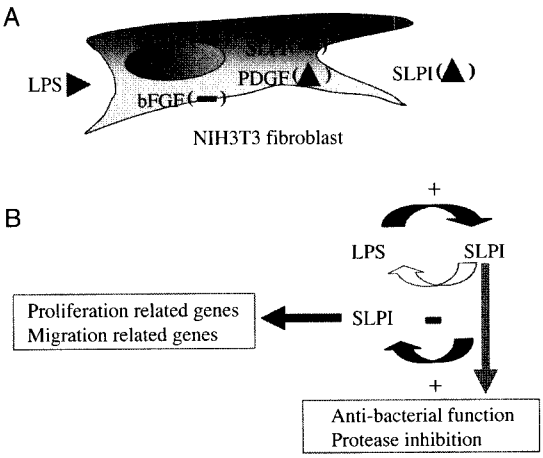

Fig. 4. Diagram of conclusions. A) mRNA expression of SLPI, VEGF, bFGF, PDGF in NIH3T3 cell after LPS stimulation. B) LPS induces the SLPI gene and protein. After induction of SLPI, secreated SLPI inhibits LPS function and released SLPI promotes proliferation and migration of neighboring cells.

LPS was already known that inhibits the proliferative activity in human gingival fibroblast (Bartold et al., 1992). However, in gingival fibroblast, LPS was used high concentration (50 μg/mL) compared to our concentration (100 ng/mL). Previous study, SLPI identified which selectively increased cyclin D1 gene expression, with the effect occurring in part at the level of promoter activity in human endometrial carcinoma cell line. Moreover cellular SLPI levels negatively influenced the anti-proliferative and pro-apoptotic insulin-like growth factor-binding protein-3 expression (Zhang et al., 2002). Therefore, secreted SLPI after induction of cytoplasmic SLPI may influence the antagonization to LPS reaction, suggesting that secreted SLPI promotes the proliferation of NIH3T3 cells (Fig. 4).

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<국문초록>

분비백혈구단백분해효소억제제 (SLPI)는 염증 부위에서 중성구 엘라스타제에 의한 손상 작용에 대해 조직을 보호하는 역할을 한다. SLPI의 새로운 기능에 대한 연구들은 단백질분해효소억제제 역할보다는 선천적 면역반응 작용에 주로 관여 할 것임을 보고하였다. 따라서 본 연구는 섬유모세포 세포주(NIH3T3)에서 박테리아성지질다당류(LPS) 자극에 의한 SLPI의 기능을 확인하기 위하여 LPS 처리에 따른 여러 성장 인자들과 비교하여 SLPI의 발현을 알아보았다. 역전사효소 중합반응(RT-PCR)과 면역학적 단백질 검출법 (Western blot)은 LPS 처리 후 SLPI와 몇몇 성장 인자들(VEGF, bFGF, PDGF)의 mRNA와 단백질의 검출을 위해 수행하였다. NIH3T3 세포주를 mL 당 100 ng의 LPS에 각각 30, 60, 90분, 24, 48 시간 동안 노출시켰다. RT-PCR 결과 SLPI와 VEGF mRNA는 LPS 처리와는 상관없이 강한 발현 양상을 보였다. bFGF mRNA는 대조군과 같이 약하게 발현하였고, PDGF mRNA는 LPS 노출 시간에 따라 점진적으로 증가하는 양상을 나타냈다. 그러나 세포질 용해액과 세포 배양액에서 SLPI 단백질의 수준은 LPS 처리에 의해 증가하였다. 또한 광학 현미경 관찰과 전자 현미경 관찰은 LPS가 NIH3T3 세포주의 형태학적인 변화를 유발시킴을 증명하였다.

따라서 LPS는 NIH3T3 세포에서 SLPI의 발현 증가를 조절하며, 분비된 SLPI는 세포분열과 이동을 자극할 것이라는 결론을 얻었다. SLPI가 세포분열과 세포이동에 어떻게 관여하는지는 아직까지 규명되지 않은 실정이므로, 추후에 SLPI 단백질이나 유전자 도입을 통하여 세포이동에 관련된 실험이 진행되어야 할 것이다.