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Stimulation of Proliferation and Migration of Oral Carcinoma Cells by SLPI

朝 鮮 大 學 校　大 學 院

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Stimulation of Proliferation and Migration of Oral Carcinoma Cells by SLPI

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A thesis submitted of the faculty of the Chosun University in partial fulfillment of the requirements for the Degree of Master of Science in the Department of Dental engineering.

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Approved by_____

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Major Advisor

SLPI에 의한 구강암세포주의 증식과 이동 촉진

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CONTENTS

LIST OF FIGURES

ABSTRACT

Stimulation of Proliferation and Migration of Oral Carcinoma Cells by SLPI

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Secretory leukocyte protease inhibitor (SLPI) is a constitutively expressed, up-regulative inhibitor of serine protease, which has the function such as the anti-bacterial and anti-fungal activity. Besides, it also involved in promoting cell proliferation and wound healing *in vitro*. Recently, SLPI has been linked to regulating the proliferation and migration of cancer cells. KB cell is a cell line derived from a human carcinoma of the nasopharynx, used as an assay for antineoplastic agents. Several studies have reported a direct correlation between SLPI expression levels and tumor progression. However there was no evidence in oral carcinoma cells. Therefore, the objective of this study is to identify whether SLPI may promote proliferation and migration of KB cell or not.

We have conducted cell viability analysis, RT-PCR, northern blot, western blot, MTT assay, migration assay, wound healing assay and immunoprecipitation. KB cell viability was not affected by serum starvation in cell viability analysis and SLPI expression was not affected by serum starvation in RT-PCR, northern blot and western blot analysis. SLPI promoted KB cell proliferation

and migration in MTT and migration assay respectively. It was also found that SLPI significantly increased wound healing and migration rate of KB cell in response to scratched artificial wounding in wound healing assay. And it was also confirmed artificial wounds induced the secretion of SLPI protein in wound healing assay.

These results indicate that SLPI may have an important role for proliferation and migration of KB cell, suggesting as a modulating molecule in oral cancer progression.

I. INTRODUCTION

Secretory leukocyte protease inhibitor (SLPI) is a constitutively expressed, up-regulatable inhibitor of serine proteases (Gipson *et al*., 1999). Human SLPI exists as a non-glycosylated, hydrophobic, cationic 12kDa protein that was originally isolated from human parotid gland secretions (Thompson & Ohlsson, 1986), consisting of two homologous cystein-rich domains of 53 and 54 amino acids (Stetler *et al*., 1986; Thompson & Ohlsson, 1986), which is not only in the salivary but also in the respiratory tract, lacrimal, and genital glands (Abe *et al*., 1991; Eisenberg *et al*., 1990; Franken *et al*., 1989). SLPI inhibits serine proteases including neutrophil elastase, trypsin, chymotrypsin, chymase, and cathepsin G (Abe *et al*., 1991; Stetler *et al*., 1986). SLPI also has the function such as the anti-bacterial and anti-fungal activity (Shugars *et al*., 1998; Sallenave *et al*., 2002). Besides, SLPI promotes wound healing (Ashcroft *et al*., 2000) and cell proliferation *in vitro* (Zhang *et al*., 2002), inhibits HIV infection (McNeely *et al*., 1995) and lysises bacteria (Hiemstra *et al*., 1996). It also was reported that SLPI has the potential of promoting the tumorigenic and metastatic of cancer cells (Devoogdt *et al.,* 2003).

Cell proliferation and migration are known as two important parts of wound healing in normal cells and these are also related with tumor progression. Tumor progression is generally associated with extensive tissue remodeling to a proper environment for tumor growth, angiogenesis and invasion. So tumor progression is generally associated with metastasis of cancer cells (Johnsen *et al*., 1998). For invasion and metastasis of tumor cell, the first and key step is cell proliferation and migration. Cell migration within a three-dimensional mass is common to many biological processes, such as wound healing and tumor cell invasion (Clow *et al*., 1999).

KB cell is a cell line derived from human carcinoma of the nasopharynx, used as a useful cell

line for antineoplastic agents (Perdue *et al*., 1982). Oral carcinoma is associated with high potential of tumor metastasis (Lee *et al*., 2007). Cell proliferation and migration are important factors in the progression and metastasis of cancers. Therefore we proposed that SLPI might behave as a tumor promoter.

Recently, SLPI has been linked to regulating the proliferation and migration of cancer cells. Several studies have reported a direct correlation between SLPI expression levels and tumor progression (Ameshima *et al*., 2000; Morita *et al*., 1999). However there was no evidence in oral carcinoma cells. SLPI is highly expressed in KB cells from our experiment. Therefore, the purpose of this study is to identify whether SLPI may promote the proliferation and migration of oral carcinoma cells or not. We attempted to demonstrate that SLPI increased not only multiplication but also migration in oral cancer cells.

II. MATERIALS AND METHODS

II-1. Cell culture

The KB (oral carcinoma cell line) cell line was cultured with Dulbecco's Modified Eagle's Medium (DMEM; Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco), 1X antibiotic antimycotic solution (AA; Gibco), 1X non-essential Amino Acids solution (NEAA; Gibco) in a humidified 5% CO_2 incubator at 37°C.

II-2. Cell viability analysis

The KB cells were plated on 60mm dishes in triplicate at a density of 2×10^5 cells per dish in the medium. After becoming confluent (control, 0h), cells were starved for 18h and 42h in serum free medium. Cell number was counted at 0h, 18h and 42h in triplicate using hemacytometer.

II-3. Reverse transcriptase polymerase chain reaction (RT-PCR)

For the lysis of KB cells, Tri reagent (MRC, Inc., Montgomery Rd, CIN, USA) was used and total RNA was extracted according to the manufacturer's protocol. A 20μL solution containing total RNA, Oligo-dT primer, dNTP mixture, and the superscript one-step reverse transcriptase (Invitrogen, Carlsbad, USA) in reaction buffer was incubated at 42°C for 60min. The solution was heated to 90°C for 5min to inactivate reverse transcriptase.

The PCR amplification was performed in a 20μL reaction mixture with published sequence

specific primers against human SLPI (Shimoya *et al*., 2006). hSLPI Sense: 5' -ACTCCTGCCTTCACCATGAA- 3', hSLPI anti-sense: 5' -CATTCGATCAACTGGCACTT- 3'. And cDNA was subjected to PCR amplification for 35 cycles. Each cycle consisted of denaturation at 94°C (40s), annealing at 52°C (40s), and extension at 72°C (40s). Before amplification, reaction mixture was incubated at 94°C for 5min. Then after amplification, extension was performed at 72°C for 5min at the last cycle. The amplification yielded a 570bp DNA product, according to the published sequence of the SLPI gene (Stetler *et al*., 1986). A housekeeping gene glyceraldehydes adenosine-phosphate dehydrogenase (GAPDH) was used as an amplification internal control for the RT-PCR assay. 15μL of PCR mixture was electrophoresed in a 1% agarose gel and stained with ethidium bromide, and the amplified products were visualized by UV illumination. Molecular size was estimated by comparison with 100bp DNA ladder.

II-4. Northern blot analysis

Total RNA from KB cells was prepared using Tri-Reagent. Total RNA (10μg) was denatured and electrophoresed in 1% agarose gel containing formaldehyde, then was transferred to a nylon membrane. After total RNA was immobilized onto a filter, hybridization was performed overnight in hybridization solution (0.5% SDS, 6X SSC,10% dextrane-sulfate, 10mM EDTA, 100μg/mL of salmon sperm DNA and the [^{32}P]-labeled insert of the indicated cDNA clone). The probe was made from Prime-It Ⅱ labeling kit (Stratagene, USA) using random primer labeling method. 10μL random oligonucleotide primer was added to 23μL of DNA template (about 25ng DNA). The tube was heated at 95°C after reaction, then the following reagents were added to the tube: 2μL of [^{32}P]-dCTP, 10μL of 5X dCTP primer buffer and 1μL of Exo(-) Klenow enzyme (5U/μL). After the reaction mixture was incubated at 37°C for 10~50min, 2μL stop mixture were added. DNA probe

was eluted in Probe Quant TMG-50 Micro Columns (Amersham Pharmacia Biotech). 5~10mL prehybridization solution containing 6X SSC, 10mM EDTA, 0.5% SDS, 10% dextran sulfate, 100μg/mL salmon sperm DNA were added to hybridization tube. Prehybridization was performed at 62°C for 2h with shaking. Then DNA probe was applied to hybridization tube and hybridization was performed at 62°C for 20h with shaking. Membranes were washed twice in 2X SSC/0.1% SDS at room temperature for 10min and once in 2X SSC/0.1% SDS at 42°C 30min, and once in 0.1X SSC/0.1% SDS at 55°C for 30min. And then the membranes were exposed to image plate for 24~48h. Then the hybridization signals were examined. The hybridization signals were examined by chemiluminescence film (Amershamphamacia, UK)

II-5. Western blot analysis

To analyze the SLPI protein expression of cytosolic form, we conducted western blotting analysis. Proteins were extracted from cell lysates by NP-40 lysis buffer containing 150mM NaCl, 1% NP-40, 50mM Tris-Cl (pH 7.4), 2mM Na_3VO_4, 2mM $Na_4P_2O_7$, 50mM NaF, 2mM EDTA (pH 7.4) and added leupeptin and aprotinin as protease inhibitor. In all the experiments, equivalent amount (20μg) of protein was loaded on to SDS 15%-polyacrylamide gels. After electrophoresis, proteins were transferred to the nitrocellulose membrane in transfer buffer (20% methanol, 25mM Tris, 40mM Glysine). After proteins were transferred, the membrane was blocked in 5% non-fat dried milk and blotted with anti-rabbit SLPI antiserum 1:500, following by goat anti-rabbit-IgG (1:10,000) (Santa Cruz biotechnology, CA, USA). The membrane was washed in phosphate-buffered-saline-Tween20 (PBS-T). The bound antibody was detected by ECL (Amershamphamacia, UK).

II-6. Immunoprecipitation (IP)

Additionally, for analyzing the secreted SLPI protein, the medium from cultured cells was used for immunoprecipitation (IP). The protein G beads slurry (KPL, Gaithersburg, MA, USA) was washed with PBS for three times and added the culture medium 500μL to the washed protein beads slurry. Anti-mouse SLPI antiserum and immobilized protein G beads were incubated together with the collected medium overnight at 4°C. After incubation, protein-bead-antibody complexes were washed with PBS and centrifuged at 10000×g for 5min. Then samples were boiled for 5min in sample buffer and loaded on to SDS 15%-polyacrylamide gels.

II-7. Migration assay

KB cells were grown to 80-90% confluence in 100mm dish and incubated with serum-free DMEM (SF media) for 24h. After washing, the cell monolayer was briefly trypsinized to lift and then was pelleted and suspend in SF media. These cells were added to FluoroBlok^{TM} cell culture inserts (Becton Dickinson Labware, NJ, USA). The inserts were used with 24-well cell culture insert companion plate. Conditioned media with human recombinant SLPI (5μg/mL, R&D Systems, Inc. USA) was used in the lower wells, while SF media to the bottom of the wells for negative control (No cells and no treatment with SLPI) and KB conditioned media to bottom of wells for positive control (no treatment with SLPI). The inserts were incubated for 4h at 37°C in 5% CO_2 incubator. The cells were then labeled with Calcein-AM (Molecular Probe, Eugene, OR, USA) in 1X phosphate buffered saline (PBS) for 30min at 37°C. After incubation, the fluorescence in the wells was measured using the Magellan software in the GENios FL (TECAN, Austria). The wavelength of fluorescence plate reader was excitation /emission; 485nm/530nm.

II-8. Wound healing assay

In vitro wound healing assays is a classic and commonly used method for studying cell proliferation and migration capacities and have been used with multiple cell types (Keese *et al*., 2004). After becoming confluent, cells were starved in a DMEM serum free medium (SF media) for 18h, then three artificial wounds per plate were scratched into the monolayer with a sterile plastic 10μL micropipette tip to generate uniform wounds that was devoid of adherent at least twice in PBS to eliminate detached cells. Experiments were performed by incubating cells with SF media only, or supplemented with 1) a concentration of 0.1μg/mL rhSLPI, b) a concentration of 1μg/mL rhSLPI for 12h and 24h, to evaluate the effect of SLPI on cell migration into the wound space. Wound healing was monitored by digital photographs taken in a phase-contrast inverted microscope (OLYMPUS, Tokyo, Japan) at the moment of wounding and 12h post-wounding and 24h post-wounding. Micrographs were analysed by image analysis program (Axiovison, Zeizz, Germany). Changes in wound size (distance between wound edges) at 12h and 24h post wounding were analyzed using the formula (wound size at 12h or 24 h /initial wound side) × 100. In order to assess the effects of SLPI on KB cell migration, the rate of cell migration was determined using the formula: (initial wound size - wound size at 12 h)/12 or (initial wound size - wound size at 24 h)/24.

II-9. Proliferation assay (MTT assay)

The increase of KB cell proliferation by SLPI was determined by the MTT assay, as described below (Twentyman *et al.*, 1989). KB cells were plated onto 96-well tissue culture dishes at a density of 5×10^{3} $well^{-1}$ in 100μL medium. After plating, cells were allowed to attach for 24h. Cells were then incubated with 5μg/mL of SLPI protein for 0h, 6h, 12h, 24h, 48h, at which time 20μL of

2 mg/mL MTT was added and the absorbance at 492nm was determined by a microtiter plate reader. The extract was performed using tetrahydrofuran (THF) as the vehicle, at a maximum concentration of 0.1%. Live cells convert 3-(4, 5- dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide to a formazan dye that can be detected at 492nm by a microplate reader. The absorbance at 690nm was also measured as a reference. The same aliquot of carotenoid-free emulsion was alone added to the control cells. Experiments were conducted in six replicates.

II-10. Trypan blue assay

For evaluation of culture cell viability and cell monolayer damage produced by 10μL micropipette tip scratch wound, KB cells were treated with 0.4% Trypan blue and immediately observed in a phase-contrast microscopy (OLYMPUS, Tokyo, Japan) across the wound area determined trypan blue exclusion (Yasui *et al*., 2003)

II-11. Statistical analysis

Data were analyzed by one-way ANOVA or t-tests with origin 7.5 statistical software (Northampton, MA, USA). The P-value of <0.05 was determined significant.

III. RESULTS

III-1. Serum starvation does not affect the viability and SLPI expression of KB cell

To exclude other factors in serum, cells were starved in serum free medium (SF medium) after being confluent. To know the effect of serum starvation, the cell viability assay was performed. Replacement of culture medium by a SF medium resulted in proliferation inhibition without alteration of cell viability, as confirmed by hemacytometer counting and Trypan blue exclusion at 0h, 18h and 42h of culture in SF media after being confluent. Cell viability was maintained 96% at 18h and 99% at 42h comparing with 0h (Fig. 1A). It indicates that serum starvation does not affect KB cell viability.

To determine whether endogenous expression of SLPI mRNA and protein in KB cells was affected or not by incubating with SF medium, total RNA and total protein were extracted from KB cell culture at 0h, 18h and 42h incubated in SF medium after being confluent. And they were subjected to RT-PCR, Northern blotting and Western blotting to determine SLPI expression. The results showed that KB cells highly expressed SLPI and there was no marked difference of SLPI mRNA and protein expression during 42h serum starvation (Fig. 1B).

III-2. SLPI increases proliferation and promotes migration of KB cells

To determine the effect of SLPI on proliferation and migration of KB cell, we conducted MTT assay and migration assay. The MTT assay results showed proliferation of SLPI treated cells was higher than control during 48h and there was a peak at 12h (Fig. 2A). As migration assay results

showed, migrated cell number in SLPI treated sample was near 3 times more than in positive control (Fig. 2B). Therefore SLPI enhanced cell migration ability.

III-3. SLPI accelerates wound healing and promotes migration rate of KB cell

After 12h and 24h of wounding, a large amount of KB cells had already migrated into the wound space. Due to active cell migration, the wound margin was observed rather diffuse in all the experiments. At the wound edges uniform cell migration was observed. The same pattern was observed when SLPI was added to the medium (Fig. 3A).

The results of wound healing assay showed that addition of 100ng/mL or 1μg/mL SLPI protein significantly increased wound healing compared to the control media (Fig. 3B). As the result of migration rate assay showed, between the time of wounding and 24h after wounding, cells treated with 100ng/mL and 1μg/mL SLPI protein supplemented media presented significantly higher migration rate at 12h as compared to the control. But at 24h the migration rate of all the samples was similar and for control medium, the migration rate of 24h is same as 12h (Fig. 3C).

III-4. Artificial wounds induce the secretion of SLPI protein

To determine whether SLPI expression was changed by wounding or not, RT-PCR, Western blot and Northern blot were performed. Total RNA and total protein were extracted from KB cells incubated with SF medium for (1) 18h after becoming confluent, and incubated with SF medium for (2) 12h and (3) 24h after wounding. And they were subjected to RT-PCR, Northern blotting and Western blotting to determine SLPI expression. The result showed that SLPI mRNA expression of SLPI was increased by artificial wound in Northern blotting results. But there was no significant

difference of SLPI expression in cell lysates in Western blotting result (Fig. 4A).

For the secreted form SLPI protein analysis, the medium from cell culture was used for IP. The results showed that when KB cells were incubated with SF medium, the secreted SLPI protein in medium was in the same amount (KBsf), but after making wound, the secreted SLPI protein in medium was increased (Fig. 4B, KBw).

IV. DISCUSSION

SLPI has been known as a potent inhibitor of leukocyte serine proteases elastase and cathepsin G from neutrophil, Chymase and Tryptase from mast cells, as well as trypsin and chymotrypsin from pancreatic acinar cells notably (Fink *et al*., 1986; Thompson & Ohlsson 1986). Besides its function as an inhibitor of inflammatory proteases, SLPI exerts pleiotropic activities in different biological systems. Such as, SLPI promotes wound healing (Ashcroft *et al*., 2000) and cell proliferation *in vitro* (Zhang *et al*., 2002). In recent years, new biological activities of SLPI were identified, such as promoting tumor growth and invasion. Several studies have reported a direct correlation between SLPI expression levels and tumor progression (Morita *et al*., 1999; Ameshima *et al*., 2000; Hough *et al*., 2000). As reported, some study have demonstrated that SLPI is up-regulated in the course of cancer development (Ameshima *et al*., 2000) and it has been identified as highly up-regulated in ovarian carcinomas (Hough *et al*., 2000). Although these studies showed that SLPI is over-expressed in tumor tissue, the significance of SLPI regulation in oral carcinoma cell has not yet been reported. In this study, we found that the expression of SLPI mRNA and protein was high in KB cell. Therefore, the function of SLPI may be related with cancer promotion in oral carcinoma cell.

There are many factors in serum and there must be some factors having relations with SLPI. And it was reported that some growth factors increased SLPI expression, such as EGF (Velarde *et al*., 2005). It was also reported that some growth factors inhibit SLPI expression, such as TGF-β_1 (Jaumann *et al*., 2000). To eliminate other effectors to SLPI and to identify the function of SLPI itself on the KB cell, we used SF medium for all experiment. In SF condition, SLPI was highly expressed in KB intact cells. Even though SLPI was highly expressed in KB cell, there was no

change of SLPI protein amount in cell lysate and secreted SLPI protein amount in medium in SF condition. Therefore, serum does not affect SLPI overall.

SLPI is produced in normal epidermis. It also is expressed in cancer tissue. But its role is not well understood. As reported previously, SLPI genes are often up-regulated under tumorigenic condition. Over-expression of SLPI has been noted in human tumors and human carcinoma cell lines of ovarian, endometrial, respiratory tract, and neural origins (Koshikawa *et al.,* 1996; Ameshima *et al.,* 2000; Hough *et al.,* 2001; Shigemasa *et al.,* 2001; Westin *et al.,* 2002). In ours study, compared with its normal cell (INOK, oral keratinocyte), KB cell's SLPI expression and proliferation are much higher (data not shown). Therefore, SLPI may be related with tumor happening

In our result, proliferation of KB cell was increased after treated with SLPI. Some people reported that SLPI inhibits cell growth on ovarian cancer. Although it is opposed with our result, several reports support that SLPI may promote proliferation of porcine endometrial glandular epithelial cells, human endometrial epithelial cells and lung carcinoma cells (Zhang *et al*., 2002; Devoogdt *et al*., 2003). For proliferation, first step is mitosis. The MAPK is believed to be an important regulator of mitogen-induced gene expression and cell cycle re-entry in the stimulation of cell proliferation (Chang and Karin 2001). MAPK is also known as extracellular signal-regulated protein kinase (ERK). The activation ERK pathway initiates cellular processes such as proliferation and cell cycle and is induced by several growth factors and mitogens and results in control of cell proliferation through stimulation of mitosis associated protein kinases (Morrison, 2001). In our experiment, SLPI inhibits phosphorylation of ERK1/2 but not changes expression of ERK1/2 (data not shown). Therefore SLPI increases proliferation in KB cell through other pathway but not ERK1/2.

In vitro wound healing assays are useful for the study of the mechanisms involved in cell

migration in multiple cell types. Although it seems a simple assay, experimental conditions differ significantly depending on the parameters to be assessed and the type of the cell line.

SLPI protein was observed during wound healing in the suprabasal epidermal cells in the migrating epidermis (Ashcroft *et al.,* 2000). It has been also reported that SLPI mRNA levels directly correlate with the metastatic potential of liver carcinoma cells (Morita *et al.,* 1999) and over-expression of SLPI was sufficient to enhance the tumorigenicity and lung-colonizing potential of 3LL-S cells(Devoogdt *et al*., 2003). In our result, after treatment of SLPI, wound area was closer and migration rate of the KB cell was faster than control (not treated SLPI). To confirm the effects of SLPI on the KB cells migration during healing, we also conducted biochemical assays. There was no difference of SLPI expression in the cell, but secreted form of SLPI in medium was increased after wounding. The temporal increase in SLPI expression during early wound healing, it suggested a role for this cytokine in repair. As supported report, SLPI expression was enhanced by wounding (Ashcroft *et al.,* 2000). It has been demonstrated that secretion of SLPI was regulated by infections or other secretion signaling factors (Si-Tahar *et al*., 2000). It has been also reported that the *in vitro* growth stimulating effect of SLPI may be from its Leu-72 **(**Devoogdt *et al*., 2003). Even though we have eliminated other factors involved in healing using SF medium, there also may be other growth factors or cytokines produced in response to wounding that could be involved in stimulating KB cell migration, which should be further analyzed. However, from increasing SLPI in the medium after wounding, it suggests that SLPI has the promoting function of KB cells migration.

In summary, the present results clearly showed that SLPI has agonistic effects for the proliferation and migration of KB cells, suggesting as a modulating molecule in oral cancer progression.

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VI. FIGURE LEGENDS

Fig. 1. Serum starvation did not affect viability and SLPI expression of KB cells

A) Cell viability was maintained 96% at 18h and 99% at 42h comparing with 0h. KB cells were incubated in DMEM medium, supplemented with 10% fetal bovine serum. After being confluent (0h), KB cells were incubated with a serum free medium for 18hours (18h) and 42hours (42h).

B) Lane 1, KB cells were incubated to confluence in the DMEM medium supplemented with fetal bovine serum, after that incubated with a serum free medium for 18h (lane2) and 42h (lane3). Total mRNA was isolated and hSLPI mRNA expression was detected by RT-PCR; Total protein was isolated and hSLPI expression was detected by western blotting. (NB, northern blotting; WB, western blotting).

Fig. 2. SLPI increased proliferation and promoted migration of KB cells.

A) The increase of KB cell proliferation by SLPI was determined by the MTT assay. The cell number of SLPI treated sample was higher than control (not treated SLPI). Cells were incubated with 5μg/mL of SLPI protein for 0h, 6h, 12h, 24h, 48h.

B) End-point analysis of Calcein-AM labeled KB cells Migration by SLPI. KB cells were incubated in the presence or absence of rhSLPI protein (5μg/mL) for 4h at 37°C in a 5% CO2 cell incubator. The * represents statistically significant differences ($p<0.05$). Data represents the mean of n=3 inserts ± SD. (RFU : relative fluorescent units, NC : negative

control, PC : positive control).

Fig. 3. SLPI promoted KB cell wound healing.

A) Effect of SLPI on KB cell migration. Representative images of cells migrating into the wounded area. KB cells were grown into monolayer in triplicate in 60mm plates. Confluent cultures were starved for 18h in DMEM medium, and then wounded with a 10μL pipette tip. There were 3 samples (0h, 12h, 24h after wounding) respectively in control, 0.1μg/mL SLPI treated and 1μg/mL SLPI treated. Scale bar, 100 μm. (Arrows: wound edge)

B) Effects of SLPI on KB cell wound healing. Wound healing photographs were taken across the wound at the moment of wounding, 12h after wounding and 24h after wounding. Wound healing was assessed as the distance between the wound edges. The percentage of wound healing was evaluated using the formula (wound side at 12h or 24 h /initial wound side)X100. The * represents statistically significant differences ($p<0.05$).

C) The migration rate (μm/h) of KB cells into the wound space was evaluated as half the distance difference between wound edges at 0h and 12h, divided by 12, or difference between wound edges at 0 and 24h, divided by 24. The * represents statistically significant differences ($p<0.05$).

Fig. 4. Artificial wounds induced the secretion of SLPI protein

A) SLPI expression was effected by artificial wound (in RT-PCR and Northern blotting), but there is not difference of SLPI expression in Western blotting result. KB cell were grown to confluence in the DMEM medium, supplemented with fetal bovine serum; after incubating with a serum free medium for 18h, we made wound (lane1). After making wound, cells incubated with a serum free medium for 12h (lane2) and 24h (lane3). (NB, northern

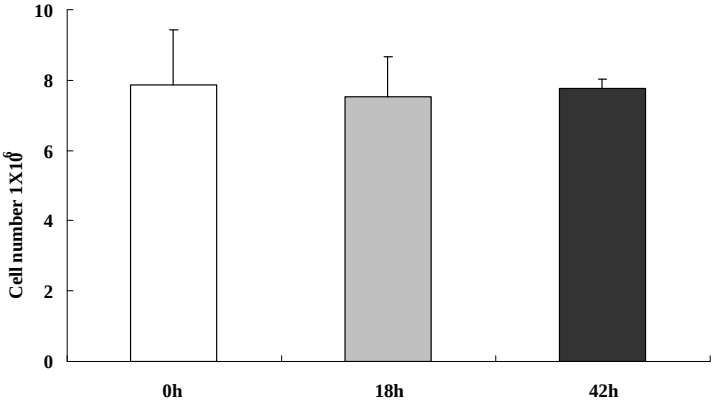
blotting; WB, western blotting).

B) Secreted SLPI protein increased after making wound. SLPI protein was detected by immunoprecipitation. The artificial wound induces SLPI protein into medium. (KBsf: serum starvation; KBw: wound)

VII. FIGURES

Figure 1.

A



B

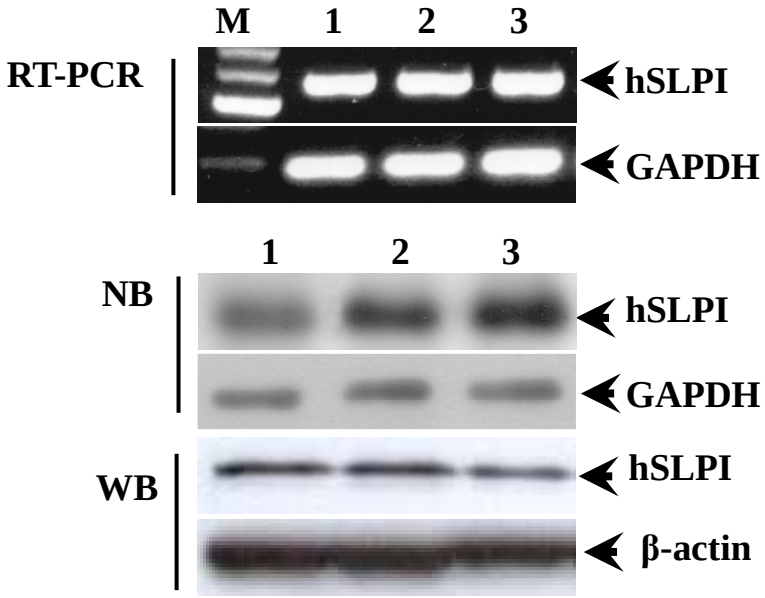
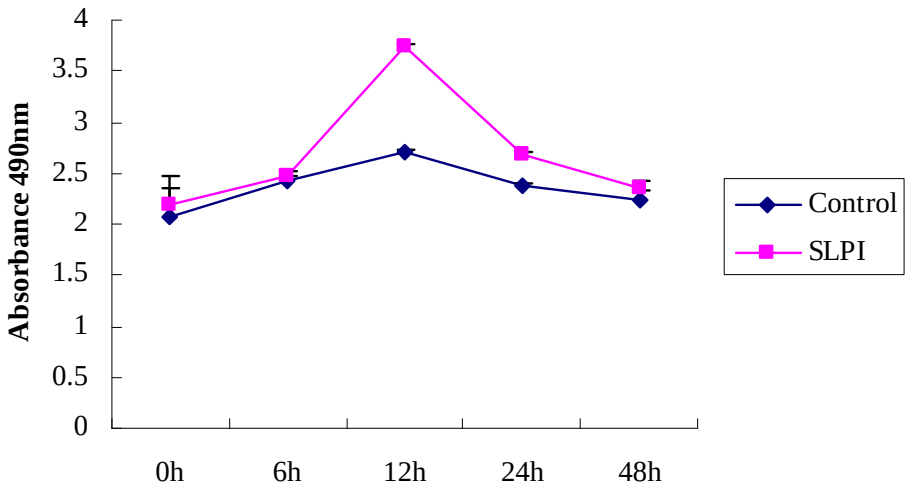


Figure 2.

A


Absorbance 490nm
4
3.5
3
2.5
2
1.5
1
0.5
0
0h
6h
12h
24h
48h
Control
SLPI

B

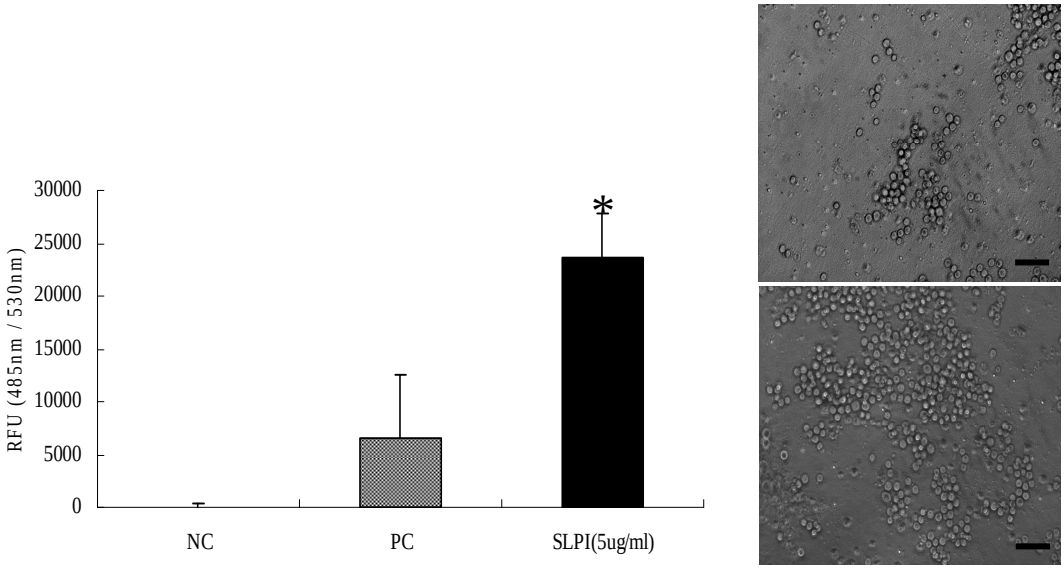
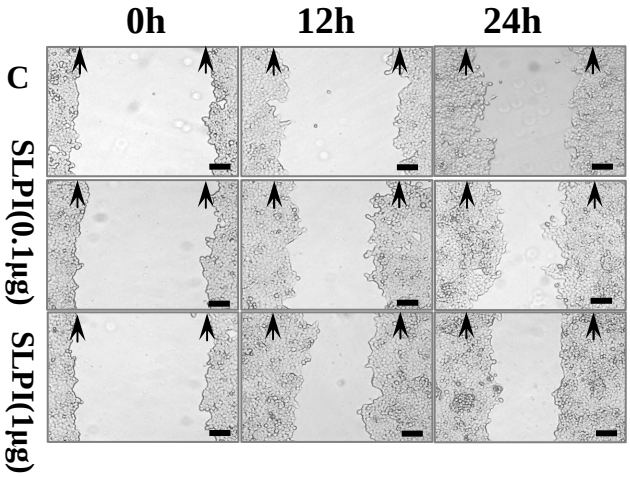
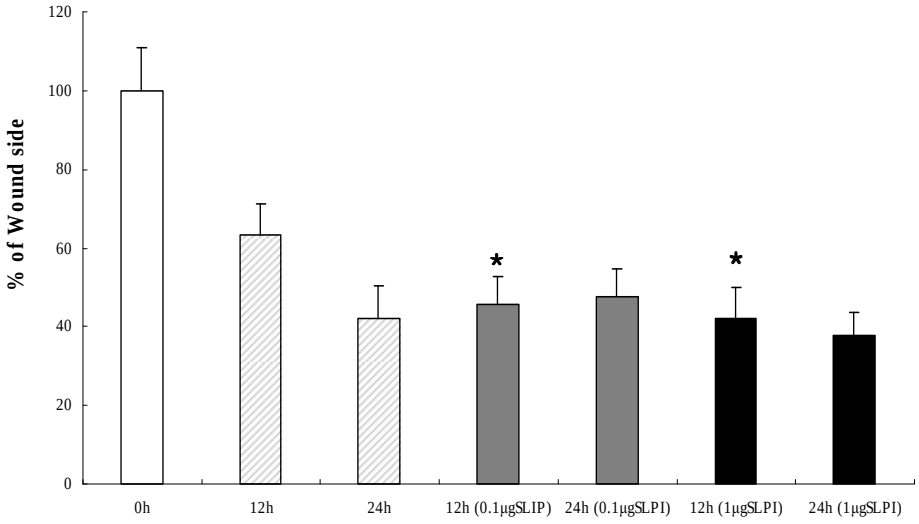

RFU (485nm / 530nm)
30000
25000
20000
15000
10000
5000
0
NC
PC
SLPI(5ug/ml)
*

Figure 3.

A


0h
12h
24h
C
SLPI(0.1μg)
SLPI(1μg)

B


% of Wound side
120
100
80
60
40
20
0
0h
12h
24h
12h (0.1μgSLIP)
24h (0.1μgSLPI)
12h (1μgSLPI)
24h (1μgSLPI)
*
*

C

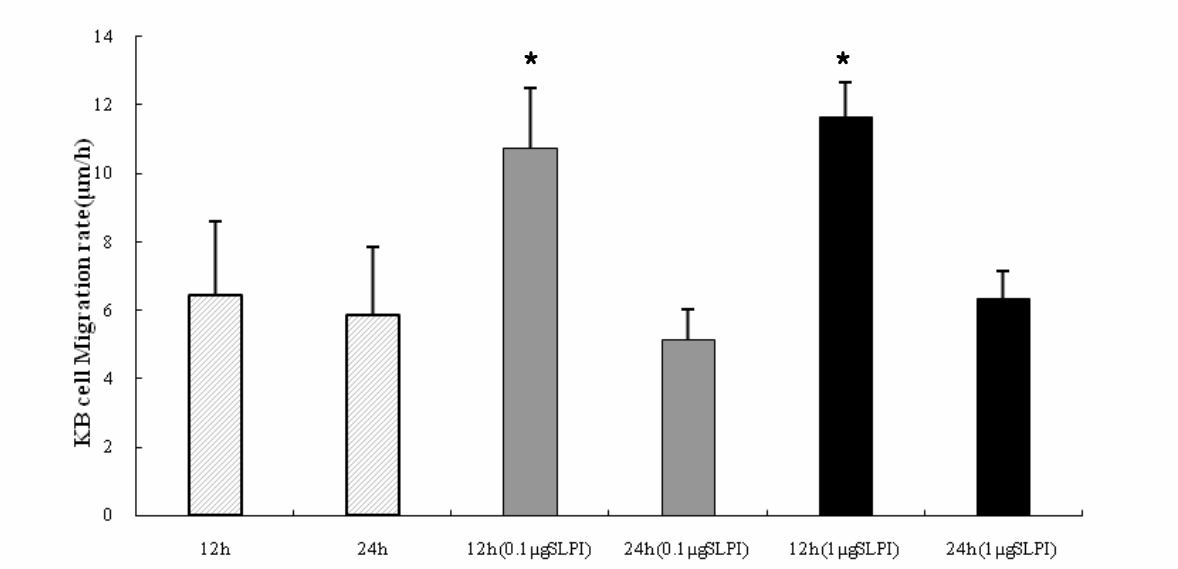
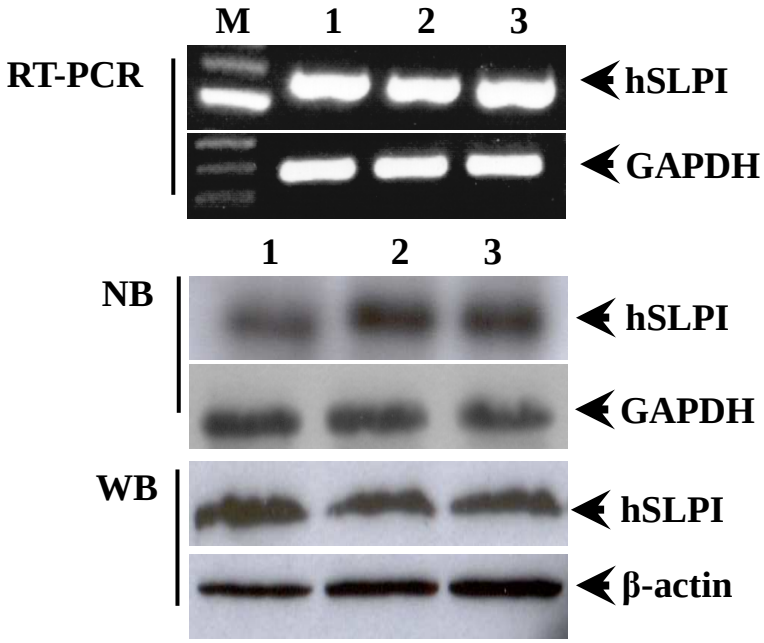
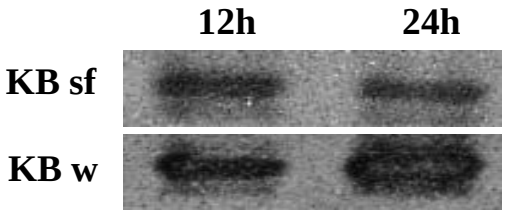

KB cell Migration rate(μm/h)
14
12
10
8
6
4
2
0
*
*
12h
24h
12h(0.1μgSLPI)
24h(0.1μgSLPI)
12h(1μgSLPI)
24h(1μgSLPI)

Figure 4.

A


M
1
2
3
RT-PCR
hSLPI
GAPDH
1
2
3
NB
hSLPI
GAPDH
WB
hSLPI
β-actin

B


12h
24h
KB sf
KB w

ABSTRACT IN KOREAN

SLPI에 의한 구강암세포주의 증식과 이동 촉진

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조선대학교 대학원 치의공학과
(지도교수: 정문진)

분비백혈구단백분해효소억제제(SLPI)는 serine 계열의 단백질을 분해하는 효소의 억제인자로서 항균 및 항진균작용을 하는 것으로 알려졌고, 더불어 세포의 증식과 상처치유를 촉진하는 것과 관계가 있는 것으로 밝혀졌다. 또한 최근에는 SLPI가 암세포의 증식과 이동을 조절한다는 연구 결과들이 보고되었고, 몇몇 연구에서는 SLPI의 발현 수준과 종양의 진행 사이에 직접적인 관계가 있다고 알려졌다. 하지만 구강암세포주의 증식 및 이동과 SLPI의 관계에 대한 연구는 거의 이루어지지 않았다. 따라서 본 연구의 목적은 비강인두의 상피세포에서 발생한 악성종양 세포주인 KB 세포를 이용해 SLPI가 구강암세포의 증식과 이동을 촉진시키는지의 여부를 알아보고자 하였다.

실험결과 혈청을 제거한 배양액에서 KB 세포는 생존율에 큰 변화가 없었고 RT-PCR, Northern blotting 그리고 Western blotting 에서도 SLPI 발현에는 변화가 나타나지 않았다. 그런데 세포증식 및 이동 실험에서 SLPI는 KB 세포의 증식과 이동을 모두 증가시키는 것으로 나타났다. 또한 상처회복 실험 결과 SLPI 단백질은 KB 세포의 상처 치유 및 그에 따른 세포 이동을 확실히 증가시켰으며, 상처가 유발된 KB 세포로부터 SLPI 단백질이 분비되는 것이 확인되었다.

따라서 본 연구의 실험 결과들은 SLPI 단백질이 구강암 진행의 조절 인자로서 KB 세포의 증식과 이동에 중요한 역할을 할 것으로 생각된다.

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X. ABBREVIATIONS

AA: antibiotic antimycotic solution

DMEM: Dulbecco's Modified Eagle's Medium

FBS: fetal bovine serum

GAPDH: glyceraldehydes adenosine-phosphate dehydrogenase

IP: immunoprecipitation

MTT: (3-(4, 5- dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide

NEAA: non-essential amino acids solution

PBS: phosphate buffered saline

SF: serum free

SLPI: secretory leukocyte protease inhibitor

RT-PCR: reverse transcriptase polymerase chain reaction

THF: tetrahydrofuran