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Effect of secretory leukocyte protease inhibitor on migration and invasion of human KB oral carcinoma cells

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Secretory leukocyte protease inhibitor (SLPI) plays an important role in promoting the invasion and metastasis of a range of cancer cells. However, there are no reports of the expression and function of SLPI in oral carcinoma cells. In this study, the oral carcinoma cell line KB was used to determine whether SLPI affects the proliferation, migration and invasion of oral carcinoma cells. RT-PCR and Western blotting revealed high levels of endogenous SLPI expression in KB cells as well as a strong increase in SLPI secretion after wounding compared to immortalized normal oral keratinocytes (INOK). The wound healing assay revealed more migration of KB cells than INOK cells, and the SLPI treatment increased the migration of KB cells. KB cell proliferation was increased significantly by the SLPI protein but decreased by SLPI-siRNA. SLPI strongly increased the migration and invasion of KB cells. On the other hand, SLPI-siRNA decreased the migration and invasion of KB cells. This suggests that SLPI plays an important role in the metastasis of oral carcinoma cells.

Keywords: SLPI; proliferation; migration; invasion; wound healing; oral squamous cell carcinoma

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. The protein consists of two homologous cysteine-rich domains containing 53 and 54 amino acids (Thompson and Ohlsson 1986), which is found in the salivary glands, respiratory tract, lacrimal and genital glands (Franken et al. 1989; Eisenberg et al. 1990; Abe et al. 1991). SLPI inhibits serine proteases, including neutrophil elastase, trypsin, chymotrypsin, chymase and cathepsin G (Abe et al. 1991). In addition, it has anti-bacterial and anti-fungal activity (Shugars and Wahl 1998; Sallenave 2002). SLPI promotes wound healing (Ashcroft et al. 2000) and cell proliferation in vitro (Badinga et al. 1999; Zhang et al. 2002), and inhibits HIV (McNeely et al. 1995) and bacterial infections (Hiemstra et al. 1996).

Cell proliferation and migration are two important aspects of wound healing in normal cells but these processes are also associated with tumor progression. Cell migration within a three-dimensional mass is common to many biological processes, such as wound healing and tumor cell invasion (Clow and McNally 1999). Tumor progression is generally associated with extensive tissue remodeling to produce a suitable environment for tumor growth, angiogenesis, invasion and metastasis of cancer cells (Johnsen et al. 1998).

Oral squamous cell carcinoma (OSCC) is associated with a high degree of invasion and potential for metastasis (Lee et al. 2007). The invasion and metastasis of OSCC has been attributed to agonistic factors in the poor microenvironment of the oral mucosa (Spiro et al. 1974). KB cells are a cell line derived from a human carcinoma of the nasopharynx, and are useful for testing antineoplastic agents (Perdue 1982). Although a number of molecules have been implicated in the metastasis of OSCC (Takes et al. 2002; Schmalbach et al. 2004), many unknown molecules remain to be discovered. Recently, SLPI has been suggested to regulate the proliferation and migration of cancer cells. Several studies reported a direct correlation between the levels of SLPI expression and

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tumor progression (Morita et al. 1999; Ameshima et al. 2000). SLPI has the potential to promote the tumorgenesis and metastasis of cancer cells (Devoogdt et al. 2003). However, there are no reports on the effects of SLPI on the proliferation, migration and invasion of oral carcinoma cells. Therefore, this study compared the effects of SLPI on the proliferation, migration and invasion of KB cells and INOK cells. RT-PCR, Western blot, wound healing, proliferation, migration and invasion assay were performed. The results demonstrated SLPI to be strongly expressed in KB cells, which leads to a high level of proliferation, migration and invasion. Therefore, SLPI promotes the proliferation, migration and invasion, leading to the metastasis of oral carcinoma cells.

Materials and methods

Cell culture and SLPI-siRNA transfection

Human KB oral carcinoma cells were purchased from the American Type Culture Collection (ATCC, USA). Immortalized normal oral keratinocytes (INOK) were generously provided by professor D.K. Kim (School of Dentistry, Chosun University, Korea). The cells (1×10^6 cells) were plated in 100-mm culture dishes and incubated in a CO_2 incubator (5% CO_2, 37°C). The KB and INOK cells were respectively cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco BRL, USA) and keratinocyte basal medium (KBM) (Lonza, USA) supplemented with a 1% antibiotic-antimycotic solution (Gibco BRL) containing 10% fetal bovine serum (Gibco BRL) and 1% non-essential amino acids solution (Gibco BRL). The following sequences were used for the synthetic human SLPI-siRNA duplexes. The forward and reverse sequences for SLPI-siRNA were 5′-GAA GUG CCA GUU GAU CAA U-3′ and 5′-AUU GAU CAA CUG GCA CUU C-3′ (Bioneer, KOR), respectively. The KB cells were subcultured in 60-mm culture dishes (1×10^5 cells) and transfected with 80 and 100 pmole (pM) of SLPI-siRNA (si-SLPI/KB) using WellFect-si^{TM} (WellGENE, KOR). The si-SLPI/KB cells were used for the proliferation, migration and invasion experiments 72 h after transfection.

Extraction of total RNA and reverse transcription and polymerase chain reaction

The total RNA was extracted from the cells using the Tri reagent (MRC Inc, USA) according to the manufacturer's instructions; 1 μg of RNA was used to synthesize the complementary DNA (cDNA). cDNA synthesis was performed using an AccuPower RT Premix (Bioneer, KOR). The PCR reaction was carried in a thermal cycler (Takara, JPN) after adding 1 μl of cDNA to the AccuPower PCR premix (Bioneer). The following primers were synthesized (Bioneer) for RT-PCR analysis: (1) human SLPI forward, 5′-ACT CCT GCC TTC ACC ATG AA-3′; reverse, 5′-CAT TCG ATC AAC TGG CAC TT-3′ (Shimoya et al. 2006); (2) glyceraldehyde 3-phosphate dehydrogenase (GAPDH) forward, 5′-CCA TGG AGA AGG CTG GG-3′; and reverse, 5′-CAA AGT TGT CAT GGA TGA CC-3′. GAPDH was used as the internal control for RT-PCR. The PCR conditions for SLPI were as follows: initial denaturation for 5 min at 94°C, followed by 29 cycles of denaturation at 94°C for 40 sec, annealing at 55°C for 40 sec and extension at 72°C for 40 sec. The PCR conditions for GAPDH were as follows: 5 min at 94°C, 30 cycles at 94°C for 30 sec, 30 sec at 56°C, and 30 sec at 72°C. After the last cycle, all the samples were incubated for an additional 5 min at 72°C. The products were electrophoresed on 1.5% agarose gel buffered with 0.5 × Tris-Borate-EDTA and stained with ethidium bromide after amplification. The staining bands were visualized by Gel-Doc (BioRad Laboratories, USA). The primer sets specifically recognized only the genes of interest, as indicated by the amplification of a single band of the expected size (570 bp for SLPI and 199 bp GAPDH) according to the nucleotide sequence of SLPI (Genbank NM_003064.2) and GAPDH (Genbank M33197.1). The intensity of the bands was measured using a Science Lab Image Gauge (FUJI FILM, JPN).

Immunoprecipitation (IP) and Western blot analysis

Western blotting was performed to analyze the expression of SLPI and β-actin protein. The total cytosolic protein was extracted using an NP-40 lysis buffer (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) 0.1 μg/ml leupeptin and 1 μg/ml aprotinin). The lysates were incubated on ice for 30 min and centrifuged at 4°C, 13,000 rpm. After protein extraction, the concentration in a 30 μg sample was determined using a *Dc* protein assay kit (BioRad Laboratories). Immunoprecipitation (IP) was used to examine the quantity of secreted SLPI protein. The cells were incubated in a culture medium without serum. Medium (500 μl) was mixed with 50 μl of a Protein G bead solution (Kirkegaard & Perry Laboratories, USA) and 1:100 of anti-rabbit SLPI (Choi et al. 2009) for 16 h at 4°C. The bead-anti SLPI complex was washed in 1 × PBS and electrophoresed onto 10–15% SDS polyacrylamide gel. After electrophoresis, the protein was transferred to a nitrocellulose membrane and blocked with 5% non-fat dry milk for 1 h at room

temperature. The membrane was blotted with 1:500 of anti-rabbit SLPI and 1:2500 of anti-mouse β-actin (SIGMA) for 16 h at 4°C. After washing, the membrane was blotted with 1:5000–1:10,000 of HRP-conjugated goat anti-rabbit or mouse-IgG (SantaCruz Biotechnology) and developed by chemiluminescence film (Amersham Pharmacia, UK) after being treated with an ECL solution (Amersham Pharmacia). The sizes of SLPI and the β-actin bands were 12 kDa and 42 kDa, respectively. The density of the expressed bands was measured using a Science Lab Image Gauge (FUJI FILM).

Wound healing assay

After the cells reached 90% confluence, they were incubated in the culture media without serum for 18 h. Three artificial wounds per plate were scratched into the monolayer using a sterile plastic 10 μl micropipette tip. After washing three times with $1 \times$PBS, the experiments were performed using serum-free media only or supplemented with 1 μg/ml recombinant human SLPI protein (rhSLPI; R&D Systems, USA) for 12 and 24 h. Wound healing was monitored by phase-contrast microscopy (Olympus, JPN) at 12 and 24 h after wounding. The images were analyzed using an image analysis program (Zeizz, GER). The rate of cell migration was determined using the following formula: (initial wound – wound at 12 h)/12 or (initial wound - wound at 24 h)/24.

Proliferation assay

The proliferation assay was performed using the method described previously (Twentyman et al. 1989) with minor modifications. The cells were plated onto 96-well plates at a density of 5×10^3 cells per well in 100 μl of the medium. After 24 h growth, the cells were incubated with or without 5 μg/ml of SLPI for 0, 6, 12, 24 and 48 h. Proliferation was assessed using an MTT assay. Six separate experiments were performed.

Migration and invasion assay

The KB, SLPI treated KB and si-SLPI/KB cells were incubated in serum-free medium for 18 h and detached from the culture plates using 25% TrypLE (Gibco BRL). The cells were transferred to serum-free DMEM medium and suspended after centrifugation for 3 min at 1000 rpm. For the migration assay, an HTS FluoroBlok™ insert (Becton Dickinson Labware, USA) was placed onto the 24-well plate and 5×10^4 cells were added to the insert. The lower chambers of a 24-well plate were filled with 500 μl serum-free DMEM medium with or without 5 μg/ml SLPI protein and the plate was incubated in a CO_2 incubator (5% CO_2, 37°C) for 4 h. The invasion assay was performed using a BioCoat™ Tumor Invasion System (BD Labware, USA) according to the manufacturer's instructions. The cells (2.5×10^4) were used in the invasion assay. The insert was coated with the basement membrane extract (BME). The lower chambers of a 24-well plate were filled with 750 μl serum-free DMEM with or without 5 μg/ml SLPI and the plate was incubated in a CO_2 incubator (5% CO_2, 37°C) for 10 h. The cells were then stained with $1 \times$PBS containing 4 μg/ml Calcein-AM (Molecular Probe, USA) for 30 min at 37°C after migration or invasion. The positive control (PC) did not contain SLPI in the lower chamber, and the negative control (NC) did not contain cells and SLPI in the insert and lower chamber, respectively. The fluorescence was measured in GENios FL (wavelength excitation 485 nm/emission 530 nm, TECAN, CHE). The cells were observed and photographed by fluorescence microscopy (Olympus, JPN).

Data analysis

All experiments were performed at least in triplicate. The data are expressed as the relative mRNA and protein levels, which were calculated as the relative ratio of the control value from arbitrary densitometry units. All the data are reported as the mean and standard deviation using the Excel 2007 statistical software (Microsoft, USA). Significant difference ($P < 0.05$) was determined using Student's *t*-test.

Results

SLPI is highly expressed in KB cells

The level of SLPI expression from the KB and INOK cells was determined by RT-PCR, Western blot and immunoprecipitation (IP). The mRNA expression of KB cells was much stronger than that of the INOK cells (Figure 1A). The endogenous and secreted levels of the SLPI protein of KB cells were much higher than those of the INOK cells (Figure 1B).

SLPI accelerates proliferation and migration of KB cells in in vitro *wounds*

Scratched wounds and a proliferation assay were performed to determine the effect of SLPI on cell migration. As shown in Figure 2A, the rate of KB cell migration increased to 181.8% and 187.6% of that observed with INOK 12 and 24 h after wounding, respectively ($P < 0.005$). SLPI (5 μg/ml) promoted KB cell migration 12 and 24 h after wounding. The rate of SLPI-treated KB cell migration increased to 173.3%

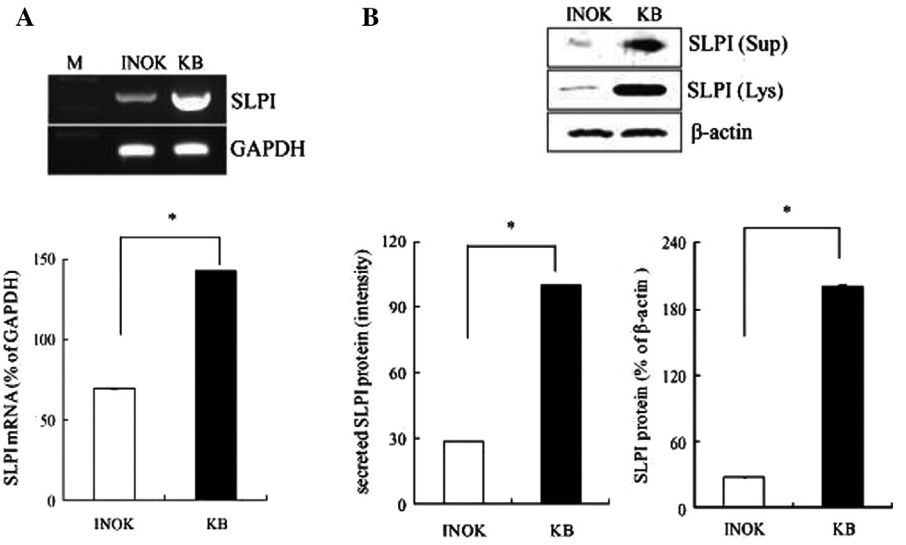

Figure 1. Expression of SLPI in INOK and KB cells. (A) The mRNA expression of SLPI from KB cells detected by RT-PCR was much stronger than that from INOK cells. (B) The levels of endogenous and secreted SLPI protein from KB cells were much higher than those of INOK cells according to Western blotting. $*P < 0.05$.

and 177.8% of the KB cells after 12 and 24 h, respectively ($P < 0.05$) but there was no significant difference between the 12 and 24 h after wounding groups.

The SLPI secreted from the INOK and KB cells during wound healing was determined by IP and Western blotting. After wounding for 12 and 24 h, the level of SLPI secreted from the INOK and KB cells increased. In particular, the SLPI secreted from the KB cells increased significantly at 24 h (Figure 2B). The proliferation assay was also performed on the INOK, KB and SLPI-treated KB and INOK cells. The MTT assay revealed an increase in the proliferation of SLPI-treated KB and INOK cells compared to the KB and INOK cells at 12 h, and the proliferation of KB cells was similar to that of the INOK cells (Figure 2C).

Transfection of SLPI-siRNA inhibits proliferation, migration and invasion of KB cells

From previous results, the increased proliferation and migration might have some association with high endogenous SLPI expression in oral carcinoma cells. To confirm this, the KB cells were transfected with SLPI-siRNA (si-SLPI/KB) at 80 or 100 pM. SLPI expression was examined by RT-PCR and Western blotting. As shown in Figure 3A, SLPI-siRNA decreased the level of SLPI expression of KB cells at 80 and 100 pM. In particular, it was decreased significantly at 100 pM. Therefore, 100 pM was used in the subsequent experiment. The level of proliferation was lower in the si-SLPI/KB cells than in the KB cells (Figure 3B). The migration assay revealed a significant increase in the migration of KB cells treated with SLPI but a decreased in the migration of si-SLPI/KB cells compared to the positive control (PC) cells. Fluorescence microscopy showed that more KB cells given the SLPI treatment migrated than the PC cells, and fewer si-SLPI/KB cells migrated than the PC (Figure 3C). The effect of SLPI on the invasion of KB cells was also examined. There was a significant increase in the invasion of SLPI-treated KB cells but a significant decrease in the invasion of si-SLPI/KB cells compared to KB cells (PC). As shown in the invasion figures, the SLPI treatment led to an invasion but SLPI-siRNA transfection impeded the invasion of KB cells compared to the PC (Figure 3D).

Discussion

Recently, SLPI was found to promote tumor growth and invasion. As reported, some studies demonstrated the upregulation of SLPI during the course of cancer development (Ameshima et al. 2000) and in ovarian carcinoma (Hough et al. 2000). The over-expression of SLPI has been reported in human tumors and carcinoma cell lines of ovarian, endometrial, respiratory tract, and neural origins (Koshikawa et al. 1996; Ameshima et al. 2000; Shigemasa et al. 2001; Westin et al. 2002). Although these studies reported that SLPI is over-expressed in different tumor tissues, there are no reports of the function of SLPI in oral carcinoma cells. Some growth factors, such as EGF (Velarde et al. 2005), were reported to increase the expression of SLPI, and TGF-β_1 was shown to inhibit the expression of SLPI (Jaumann et al. 2000). In particular, KB cells possess highly expressed

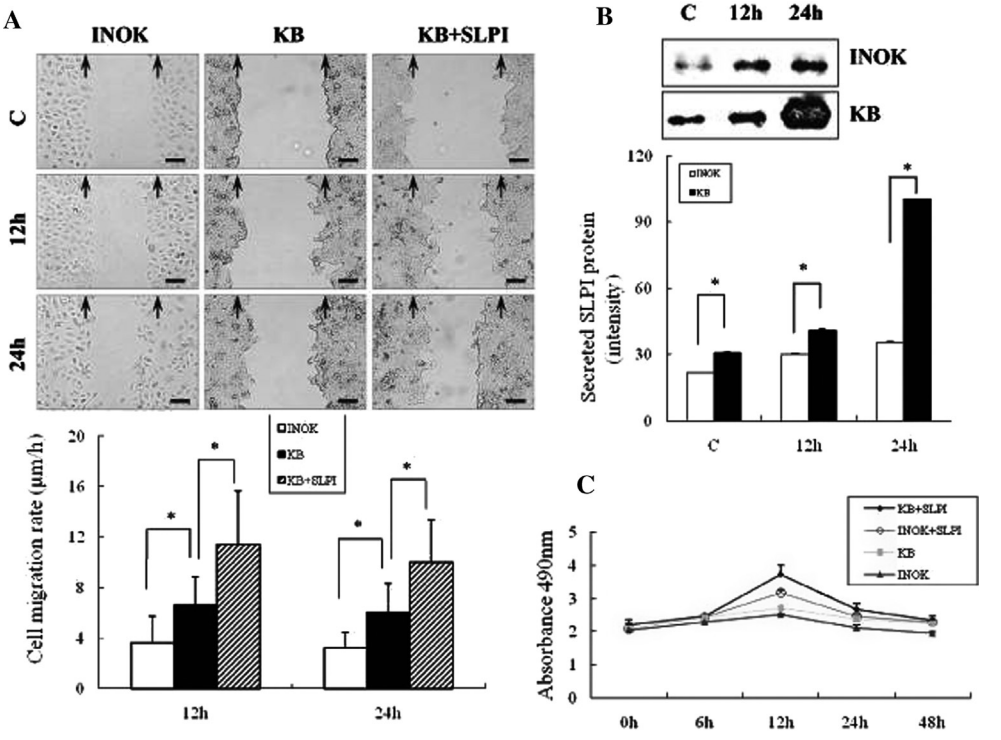

Figure 2. Wound healing and proliferation assay of INOK, KB and SLPI-treated KB cells. (A) Microscopy images show the differences in the wound area of INOK, SLPI-treated and untreated KB cells. Scale bars, 100 μm. C, control; arrows, wound edge. The rate of KB cell migration was higher than that of INOK cells and the rate of SLPI-treated KB cell migration was higher than that of KB cells. (B) Secreted SLPI was increased significantly in the medium of KB cells compared with that of INOK cells after wounding. (C) Proliferation of KB cells was similar to that of INOK cells but that of SLPI (5 μg/ml) treated KB and INOK cells was promoted. $*P < 0.05$.

epidermal growth factor receptors (EGFR) (Nicolini et al. 1996). Therefore, serum-free medium was used to detect the precise expression of SLPI in all experiments because the serum contains many growth factors that may stimulate SLPI expression. In this study, KB cells showed much stronger SLPI expression than INOK cells. These results are consistent with the reported high basal expression of SLPI in carcinoma cells and transformed tissue (Shigemasa et al. 2001; Devoogdt et al. 2003). Therefore, the strong expression of SLPI might be related to cancer progression in oral carcinoma cells.

SLPI is expressed in migrating cells of the suprabasal epidermis during wound healing (Ashcroft et al. 2000). The induction of SLPI mRNA correlates directly with the metastatic potential of liver carcinoma cells (Morita et al. 1999). Moreover, the over-expression of SLPI is sufficient to enhance the tumorigenicity and lung-colonizing potential of 3LL-S cells (Devoogdt et al. 2003). Based on these reports, an in vitro wound healing assay was carried out to determine the function of SLPI on migration. Cell migration during wound healing was demonstrated by an increase in the migration of SLPI-treated KB cells compared to the untreated KB cells and INOK cells. Some studies reported that SLPI had autocrine effects (Badinga et al. 1999) and the secretion of SLPI was induced by wounding, LPS and EGF (Ashcroft et al. 2000; Velarde et al. 2005; Lee et al. 2006). Although the expression of SLPI in cells is similar, the amount of SLPI secreted was increased by LPS stimulation (Lee et al. 2006). Therefore, this study examined the secreted form of SLPI in the medium, which was increased significantly after wounding. The results suggest that SLPI can promote the migration of oral carcinoma cells.

Although SLPI is expressed restrictedly in normal cells and tumor cells in specific organs (Franken et al. 1989; Eisenberg et al. 1990; Abe et al. 1991), the expression of SLPI is increased in squamous cell carcinoma of the lung (Ameshima et al. 2000). On the other hand, another report showed that SLPI is expressed at low levels in the squamous metaplastic epithelium (Aarbiou et al. 2004). Recently, it was reported that SLPI is involved in EGF-stimulated cell proliferation, and EGFR signaling increases the level of SLPI (Velarde et al. 2005). In addition, SLPI expression is increased by EGF and correlates with the rate of keratinocyte growth (Lai et al. 2004). In this study, the proliferation of KB cells was increased by the

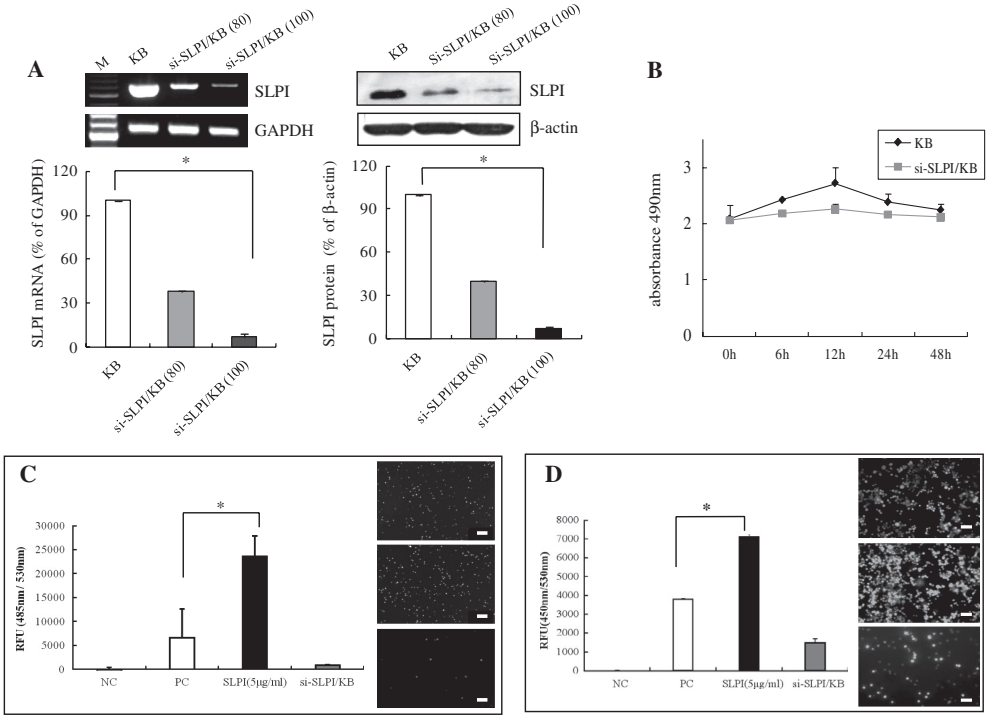

Figure 3. Proliferation, migration and invasion assay of KB cells treated with SLPI or transfected with SLPI-siRNA. (A) Expression of mRNA and protein of SLPI was decreased in si-SLPI/KB cells at 80 or 100 pM. (B) The proliferation of si-SLPI/KB cells (100 pM) was decreased compared to that of KB cells. (C, D) The migration and invasion of SLPI-treated KB cells was increased but si-SLPI/KB (100 pM) was decreased compared to that of KB cells. Scale bars, 100 μm. RFU, relative fluorescent units; NC, negative control; PC, positive control; SLPI (5 μg/ml), KB cells treated with 5 μg/ml SLPI; si-SLPI/KB, KB cells transfected with SLPI-siRNA; $*P < 0.05$.

SLPI protein but decreased by SLPI-siRNA. This is supported by reports showing that SLPI can promote the proliferation of porcine endometrial glandular epithelial cells, human endometrial epithelial cells and lung carcinoma cells (Badinga et al. 1999; Ameshima et al. 2000; Zhang et al. 2002).

In general, the invasion of cancer cells is believed to be an essential activity in the metastatic process (Chambers et al. 2002). Although the molecular mechanisms involved in the promotion of metastasis by SLPI have not been clarified, many reports have shown that SLPI can enhance tumor growth and metastasis (Ameshima et al. 2000; Zhang et al. 2002; Devoogdt et al. 2003). In this study, the SLPI-treated KB cells showed a strong increase in migration and invasion but the migration and invasion of KB cells were reduced by SLPI-siRNA, supporting the results of the in vitro wound healing assay as well as the agonistic effect of SLPI on cancer development (Devoogdt et al. 2003, 2004). Another study reported similar findings in that the down-regulation of SLPI expression reduces the capacity to form tumors in 3LL-S cells (Devoogdt et al. 2006). In summary, these results showed that SLPI increases the proliferation, migration and invasion of KB cells, which suggests that SLPI is involved in the metastasis of oral carcinoma cells.

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