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Antimetastatic activity of pinosylvin, a natural stilbenoid, is associated with the suppression of matrix metalloproteinases☆

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Abstract

Metastasis is a major cause of death in cancer patients. Our previous studies showed that pinosylvin, a naturally occurring *trans*-stilbenoid mainly found in *Pinus* species, exhibited a potential cancer chemopreventive activity and also inhibited the growth of various human cancer cell lines via the regulation of cell cycle progression. In this study, we further evaluated the potential antimetastatic activity of pinosylvin in *in vitro* and *in vivo* models. Pinosylvin suppressed the expression of matrix metalloproteinase (MMP)-2, MMP-9 and membrane type 1-MMP in cultured human fibrosarcoma HT1080 cells. We also found that pinosylvin inhibited the migration of HT1080 cells in colony dispersion and wound healing assay systems. In *in vivo* spontaneous pulmonary metastasis model employing intravenously injected CT26 mouse colon cancer cells in Balb/c mice, pinosylvin (10 mg/kg body weight, intraperitoneal administration) significantly inhibited the formation of tumor nodules and tumor weight in lung tissues. The analysis of tumor in lung tissues indicated that the antimetastatic effect of pinosylvin coincided with the down-regulation of MMP-9 and cyclooxygenase-2 expression, and phosphorylation of ERK1/2 and Akt. These data suggest that pinosylvin might be an effective inhibitor of tumor cell metastasis via modulation of MMPs.

Keywords: Pinosylvin; Antimetastasis; Matrix metalloproteinase (MMP); Cyclooxygenase-2 (COX-2); Pulmonary metastasis model

1. Introduction

Metastasis, the migration process of malignant tumor cells from the primary neoplasm to the distant secondary organs or tissues, is known as a major cause of cancer mortality. Metastasis proceeds through several consecutive steps including neovascularization, migration, intravasation, circulation of lymphatic or vascular channels, extravasation and ultimate metastatic colonization [1]. It is conceivable that the infiltration of extracellular matrix (ECM) is a major physiological barrier for malignant cells in the metastasis. Therefore, zinc-dependent proteinases matrix metalloproteinases (MMPs) that are involved in the degradation of ECM might be a promising target for antimetastasis [2,3]. In normal physiology, MMPs are involved in embryonic development, wound healing, ovulation, bone remodeling and immune cell migration [4]. However, aberrant activation of MMPs and resultant degradation of ECM can cause serious disorders including rheumatoid arthritis, periodontal disease, osteoarthritis, gastric ulcer, arteriosclerosis as well as tumor metastasis [1]. Therefore, MMPs with degradation activities on ECM have received extensive attentions as target molecules against metastasis. Indeed, compounds with inhibitory effects on MMPs have been suggested as potential antimetastatic agents [5–8]. In addition, cyclooxygenase-2 (COX-2), one of the inducible enzymes in the host defense system and in the biosynthesis of prostaglandins, is frequently up-regulated in transformed cells as well as in malignant tissues. Moreover, the positive correlation between COX-2 and metastasis has been reported in many studies in which selective COX-2 inhibitors including celecoxib, etodolac and NS-398 can suppress metastasis as well as the expression of COX-2 itself observed in various malignant lesions [9–11].

Naturally occurring stilbenoids have been reported to possess various biological and pharmacological activities such as antioxidant, anti-inflammatory and anticarcinogenic effects [12–15]. Pinosylvin (3,5-dihydroxy-*trans*-stilbene), mainly found in heartwoods and leaves of *Pinus* species, is also one of the naturally occurring stilbenoids. Pinosylvin has been known as a phytoalexin generated through the reaction between cinnamoyl-CoA and malonyl-CoA in the condition of environmental stresses such as fungal attacks,

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ultraviolet light, ozone or desiccation [16]. To date, pinosylvin has been reported to possess algicidal, antifungal, antineoplastic, antifeedant, nematocidal and gastric hydrogen ion-potassium ATPase inhibiting activities [17–19]. However, the cancer chemopreventive effect and the anticancer effect of pinosylvin have been poorly elucidated. In the present study, we report for the first time that pinosylvin has an antimetastatic potential in *in vitro* and *in vivo* models by modulating MMPs.

2. Materials and methods

2.1. Chemicals and reagents

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), antibiotics–antimycotics solution (100 U/ml penicillin G sodium, 100 μg/ml streptomycin sulfate and 0.25 μg/ml amphotericin B) and trypsin-EDTA were purchased from Invitrogen Co. (Carlsbad, CA, USA). Bicinchoninic acid (BCA), human type I collagen, gelatin, trichloroacetic acid, sulforhodamine B, dimethyl sulfoxide (DMSO), mouse monoclonal anti-β-actin antibody, TRI reagent and Bouin's solution were purchased from Sigma-Aldrich (St. Louis, MO, USA). Coomassie Blue R-250 and agarose were purchased from Amresco Inc. (Solon, OH, USA). Goat polyclonal anti-COX-2, rabbit polyclonal anti-ERK1/2 and mouse monoclonal anti-p-ERK1/2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse polyclonal anti-MMP-9 was purchased from Calbiochem (San Diego, CA, USA). Rabbit polyclonal anti-p-Akt (Tyr308) and rabbit polyclonal anti-Akt were purchased from Cell Signaling Biotechnology (Danvers, MA, USA).West Save HRP-chemiluminescent detection kit was obtained from Lab Frontier (Seoul, Korea). Gene-specific primers were synthesized by Bioneer (Daejeon, Korea). AMV reverse transcriptase, dNTP mixture, random primer, RNasin and Taq polymerase were purchased from Promega (Madison, WI, USA). Pinosylvin was prepared by the chemical synthesis and enzymatic biotransformation described previously [20].

2.2. Cell culture

One of the highly metastatic cell lines, HT1080 human fibrosarcoma cells that contain an activated N-ras oncogene and produce relatively large amount of MMPs were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin G sodium, 100 μg/ml streptomycin sulfate and 0.25 μg/ml amphotericin B at 37°C in 5% CO_2 in a humidified incubator.

2.3. Reverse transcription–polymerase chain reaction (RT-PCR) analysis

After treatment with test compounds for 24 h, total RNA of cells was extracted using TRI reagent according to the method of Chomczynski and Sacchi [21]. The extracted RNA (1 μg) was reverse transcribed using AMV reverse transcriptase, and produced cDNA was amplified by GoTaq DNA polymerase with the oligonucleotides described in Table 1. β-Actin was used as an internal standard. The PCR products were resolved on 2% agarose gel electrophoresis, and DNA bands were visualized by SYBR Gold staining.

2.4. Real-time RT-PCR

Real-time RT-PCR was employed to determine the gene expression of MMPs in human fibrosarcoma cells. Briefly, HT1080 cells (2×10^5 cells/ml) were cultured in 60-mm dish for 24 h. At almost confluency, the cells were treated with pinosylvin (12.5, 25 or 50 μM) in serum-free media for 24 h. Total cellular RNA was extracted with TRI reagent and reverse transcribed at 42°C for 60 min in 20 μl of reverse transcription system (Promega, MI, USA) with 0.5 μg of oligo$(dT)_{15}$ primer. Specific MMP primers were designed using Roche Applied System (Basel, Switzerland) and custom synthesized by Bioneer Corporation (Daejon, Korea). The following sequences were used: MMP-2 forward, 5′-ATAACCTGGATGCCGTCGT; MMP-2 reverse, 5′-AGGCACCCTTGAAGAAGTAGC-3′; MMP-9 forward, 5′-GAACCAATCTCACCGACAGG-3′; MMP-9 reverse, 5′-GCCACCCGAGTGTAACCATA-3′; β-actin (housekeeping gene) forward, 5′-AGCACAATGAAGATCAAGAT-3′; β-actin reverse, 5′-TGTAACGCAACTAAGTCATA-3′. Real-time PCR was conducted on an MiniOpticon system (Bio-Rad, Hercules, CA, USA) using 5 μl of reverse transcription product, iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) and primers in a total volume of 20 μl. The standard thermal cycler conditions were employed: 95°C for 20 s before the first cycle; 95°C for 20 s, 56°C for 20 s and 72°C for 30 s repeated 40 times; followed by 95°C for 1 min and 55°C for 1 min. The threshold cycle (C_T), indicating the fractional cycle number at which the amount of amplified target gene reaches a fixed threshold from each well, was determined using by MJ Opticon Monitor software. Relative quantification, representing the change in gene expression from real-time quantitative PCR experiments between sample-treated group and untreated control group, was calculated by the comparative C_T method as published earlier [22]. The data were analyzed using the equation $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T=[C_T \text{ of target gene}-C_T \text{ of housekeeping gene}]_{\text{treated group}}-[C_T \text{ of target gene}-C_T \text{ of housekeeping gene}]_{\text{untreated control group}}$. For the treated samples, evaluation of $2^{-\Delta\Delta C_T}$ represents the fold change in gene expression, normalized to a housekeeping gene (β-actin) and relative to the untreated control.

Table 1
Sequences of the primer pairs of specific target genes used in RT-PCR

Target gene	Sequences	
MMP-2	Sense	5′-AGA TCT GCA AAC AGG ACA TTG TAT T-3′
	Antisense	5′-TTC TTC TTC ACC TCA TTG TAT CTC C-3′
MMP-9	Sense	5′-CTG GGC TTA GAT CAT TCC TCA GT-3′
	Antisense	5′-AGT ACT TCC CAT CCT TGA ACA AAT A-3′
MT1-MMP	Sense	5′-GGG CCT GCC TGC GTC CAT CAA CA-3′
	Antisense	5′-GCC GCC CTC CTC GTC CAC CTC AAT-3′
TIMP-1	Sense	5′-TGG GGA CAC CAG AAG TCA AC-3′
	Antisense	5′-TTT TCA GAG CCT TGG AGG AG-3′
TIMP-2	Sense	5′-GTC AGT GAG AAG GAA GTG GAC TCT-3′
	Antisense	5′-ATG TTC TTC TCT GTG ACC CAG TC-3′
β-Actin	Sense	5′-AGC ACA ATG AAG ATC AAG AT-3′
	Antisense	5′-TGT AAC GCA ACT AAG TCA TA-3′
Mouse COX-2	Sense	5′-GGAGAGACTATCAAGATAGTGATC-3′
	Antisense	5′-ATGGTCAGTAGACTTTTACAGCTC -3′
Mouse β-actin	Sense	5′-TGTGATGGTGGGAATGGGTCAG -3′
	Antisense	5′-TTTGATGTCACGCACGATTTCC -3′

2.5. Wound healing assay

To assess the motility of cells exposed to test compounds, wound healing assay was performed. HT1080 cells were seeded in six-well plates at a density of 1×10^5 cells/well and incubated for 48 h. A confluent monolayer of HT1080 cells was artificially wounded with a pipette tip, and the detached cells were washed with serum-free DMEM and treated with test compounds in 1% FBS-DMEM for 17 h. The cells were fixed with 4% paraformaldehyde for 30 min and stained with hematoxylin and eosin solution. Wounds were photographed under the inverted microscope.

2.6. Colony dispersion assay

HT1080 cells (50×10^4 cells/ml, 20 μl) were loaded on the center of a 24-well plate. After 6 h of incubation, cells were incubated with 10% FBS-DMEM overnight and then treated with test compounds for 48 h. Cells were fixed with methanol and stained with hematoxylin and eosin, followed by photographing.

2.7. In vivo pulmonary metastasis method

2.7.1. Animals

Five-week-old Balb/c male mice were purchased from Central Laboratory Animal (Seoul, Korea) and acclimated for 1 week under 22°C±2°C, a 12-h light/dark cycle and a pathogen-free environment. All animal experiments and care were conducted in a manner conforming to the Guidelines of the Animal Care and Use Committee of Ewha Womans University approved by the Korean Association of Laboratory Animal Care.

2.7.2. Cell culture

CT26 mouse colon cancer cells were purchased from Korean Cell Line Bank (Seoul, Korea) and cultured in DMEM supplemented with 10% heat-inactivated FBS and antibiotics–antimycotics at 37°C with 5% CO_2 in a humidified air.

2.7.3. Experimental design

CT26 cells are an *N*-nitroso-*N*-methylurethane-induced transplantable colon carcinoma cell line and known to be highly tumorigenic [23], and cause lung metastasis when intravenously injected. A pulmonary colonization assay was performed as described by Fidler [24]. In this study, CT26 cells were diluted in PBS at a density of 1×10^5 cells per 200 μl (cell suspension) and injected into tail vein of each male Balb/c mouse (6 weeks old). After 3 h, test compounds dissolved in 2% DMSO in PBS were administered intraperitoneally with a daily dose of 2 mg/kg or 10 mg/kg for 10 days ($n=11$ per group). On day 15 after cell injection, the mice were sacrificed, and the lungs were harvested. Lung weight of each mouse was measured and fixed with Bouin's solution overnight. The numbers of peripheral nodules of each lung (five lobes) were counted using a magnifier.

2.7.4. Western blot analysis

The lung tissues were ground with an ice-cold 1× non-reducing RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 0.1% sodium dodecyl sulfate (SDS), 50 mM NaF, 50 mM Na_3VO_4 and protease inhibitor cocktail) and incubated for 40 min on ice. The lysates were centrifuged at 12,000*g* for 30 min, and the supernatant was collected and stored at −80°C. The total protein contents of the lung tissue with tumor nodules were determined using the BCA method. The proteins (30 μg) were denatured by boiling with a lysis buffer (250 mM Tris-HCl, pH 6.8, 4% SDS, 10% glycerol, 0.006% bromophenol blue, 2% β-mercaptoethanol and 2 mM sodium *ortho*-vanadate)

for 5 min. The same amount of protein in each lysate was loaded and separated by SDS–polyacrylamide gel electrophoresis and then electrotransferred to PVDF membrane. After blocking of nonspecific binding with 5% skimmed milk in PBS, membranes were incubated with primary antibodies and subsequently corresponding HRP-conjugated antibodies. The protein was detected by West Save HRP-chemiluminescent detection kit using LAS-3000 Imager (Fuji Film Corp., Tokyo, Japan).

2.8. Gelatin zymography

MMP-2 and MMP-9 enzymatic activities were assessed by gelatin zymography [25]. Briefly, the protein lysates extracted from lung tissues were denaturated by mixing 5× gel loading buffer containing 0.1 M Tris (pH 6.8), 50% glycerol, 2% SDS and 0.1% bromophenol and electrophoretically separated on a polyacrylamide gel containing 0.2% gelatin. The resolved proteins in the gel were washed and renatured by exchange of SDS with nonionic detergent Triton X-100 containing washing buffer (50 mM Tris–HCl, pH 7.5, 100 mM NaCl and 2.5% Triton X-100) and further incubated with incubation buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10 mM $CaCl_2$, 0.02% NaN_3, 1 μM $ZnCl_2$) for 24 h at 37°C with shaking. The incubated gel was stained with Coomassie Blue R-250, and proteolytic activities of MMPs were detected as clear bands with degradation of gelatin against a blue background.

In addition, HT1080 cells (2.5×10^5 cells/ml) were seeded in 24-well plates and incubated for 48 h. At 80% confluency, the cells were treated with pinosylvin (12.5, 25 or 50 μM) in serum-free media for 72 h. The culture supernatant was used for MMP-2 and MMP-9 enzymatic activities by gelatin zymography.

2.9. Statistical analysis

Data are expressed as means±S.D. Statistical analysis of data was performed using SigmaStat 2.03 (SPSS, Chicago, IL, USA). One-way analysis of variance (ANOVA) was used to compare mean responses among the treatments. Statistical probability of $*P<.05$ was considered significant. Band intensity was measured using ImageJ software from the National Institutes of Health [26] and presented as a fold change in comparison with control after the normalization to internal standard β-actin.

3. Results

3.1. Effect of pinosylvin on the mRNA expression of MMPs and TIMPs in HT1080 fibrosarcoma cells

To determine whether pinosylvin might affect metastasis in highly metastatic cancer cells, the effect of pinosylvin on the mRNA expression of metastasis-related molecules was examined by using RT-PCR and real-time RT-PCR method. As a result, in RT-PCR analysis, the treatment with various concentrations of pinosylvin (12.5, 25 or 50 μM) down-regulated the mRNA expression of MMP-9, MMP-2, membrane type 1 (MT1)-MMP and TIMP-1, whereas the mRNA expression of TIMP-2 was not changed by pinosylvin in HT1080 cells (Fig. 1A). To further determine the quantitative expression of main MMPs such as MMP-2 and MMP-9, real-time RT-PCR analysis was performed in HT1080 cells. Pinosylvin remarkably suppressed the MMP-2 gene expression in a concentration-dependent manner (Fig. 1B). However, the suppression of MMP-9 gene expression was found in a relatively high concentration of pinosylvin (50 μM). These results suggest that the inhibition of metastasis is in part associated with the down-regulation of MMP-2 and MMP-9 gene expression.

3.2. Suppressive effects of pinosylvin on MMP-2 and MMP-9 enzymatic activity in HT1080 fibrosarcoma cells

Base on the suppressive effect of pinosylvin on the gene expression of MMPs, further study was designed to investigate whether pinosylvin affects the enzymatic activity of MMP-2 and MMP-9. HT1080 human fibrosarcoma cells were treated with the pinosylvin (12.5, 25 or 50 μM) for 3 days, and then the enzymatic activity was determined by gelatin zymographic analysis. As shown in Fig. 2, the MMP-2 and MMP-9 proteins were constitutively expressed in untreated control cells, but the treatment of pinosylvin markedly suppressed the MMP-2 and MMP-9 protein expressions in a concentration-dependent manner.

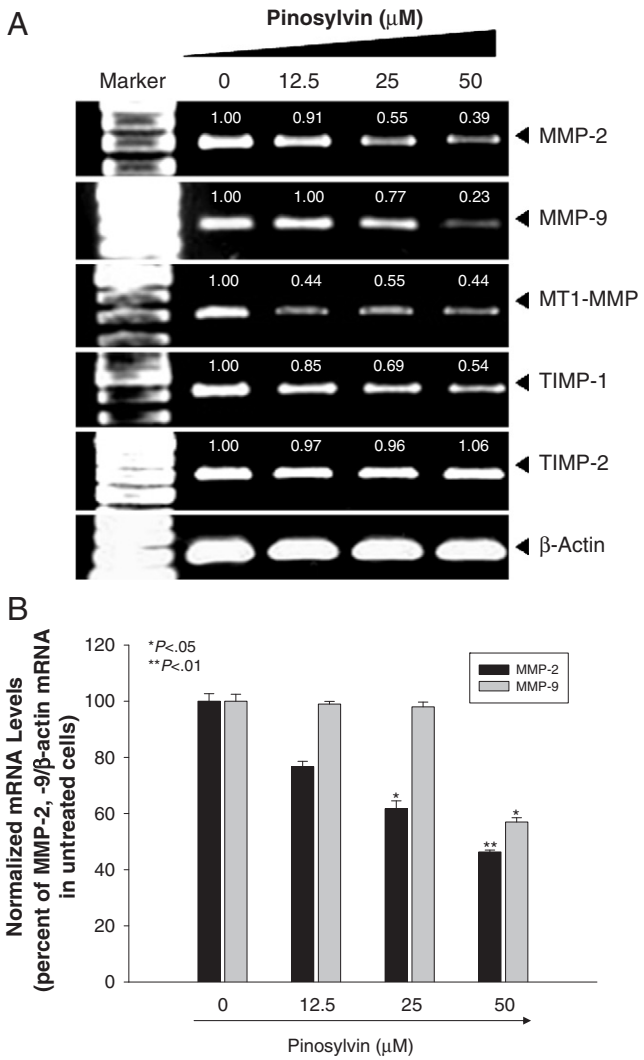

Fig. 1. (A) Effect of pinosylvin on the mRNA expression of MMP-9, MMP-2, MT1-MMP, TIMP-1 and TIMP-2. HT1080 cells were incubated with various concentrations of pinosylvin (12.5, 25 or 50 μM) diluted in serum-free DMEM for 24 h. After incubation, total RNA (1 μg) was extracted and used in the RT-PCR. cDNA products amplified with specific primers were separated on a 2% agarose gel, stained with SYBR Gold and visualized under UV illumination. β-Actin was used as an internal standard. Data are representative of three independent experiments ($n=3$). (B) The mRNA levels of MMP-2 and MMP-9 were determined by real-time RT-PCR as described in Materials and methods. Statistical analyses were performed using the Student's *t* test and one-way ANOVA. Each value represents the mean±S.D. for three different experiments performed in triplicate. $*P<.05$ and $**P<.01$ were considered statistically significant.

3.3. Inhibitory effect of pinosylvin on the migration of HT1080 fibrosarcoma cells

Cellular migration is one of fundamental features of cancer metastasis. On the basis of the mRNA expression data (Fig. 1A and 1B), to confirm the antimetastatic potential of pinosylvin, colony dispersion and wound healing assays were performed to examine the effect on the migratory features of HT1080 fibrosarcoma cells. In colony dispersion assay, control cells scattered radically from the center after treatment for 48 h. However, the treatment with pinosylvin (10 μM) resulted in the suppression of cell dispersion with a decrease in diameter (Fig. 3A). In wound healing assay, control cells migrated toward the scratched region, resulting in 'wound healing' with a narrow margin. On the other hand, cells treated with various concentrations of pinosylvin (5, 10 or 20 μM) concentration-dependently inhibited the migration of cells (Fig. 3B).

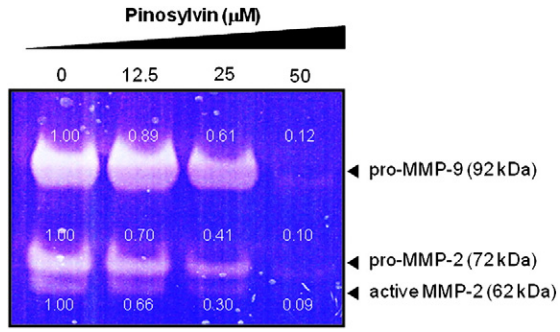

Fig. 2. Effects of pinosylvin on the activities of MMP-2 and MMP-9 in HT1080 cells. HT1080 cells were treated with various concentrations of pinosylvin. After 72 h of incubation, conditioned media were collected, and gelatin zymographic analysis was performed.

3.4. Inhibitory effect of pinosylvin on in vivo pulmonary metastasis

On the basis of the *in vitro* antimetastatic potential of pinosylvin, to better understand the effect of pinosylvin on cancer metastasis, *in vivo* pulmonary metastasis model was performed in male Balb/c mice. Fifteen days after injection of CT26 cells, all mice were sacrificed and analyzed. The CT26 cells-injected control mice were shown to have an increase of 80% in lung weight (1.0±0.3 g) with the formation of nodules (472±28), compared to the normal control mice, which were recorded with an average lung weight of 0.2±0.01 g without any nodules. Intraperitoneal injection of pinosylvin at doses of 2 and 10 mg/kg/day for 10 days attenuated the increase of both lung weight by 19% (0.8±0.2 g) and 54% (0.4±0.2 g) and the formation of nodules by 8% (436±38) and 46% (254±34), respectively. Especially, mice treated with a high dose of pinosylvin (10 mg/kg/day) demonstrated significant differences in both lung weight and number of tumor nodules compared to CT26 control mice (P<.01) (Fig. 4). No overt toxicity or bodyweight change was found with the treatment

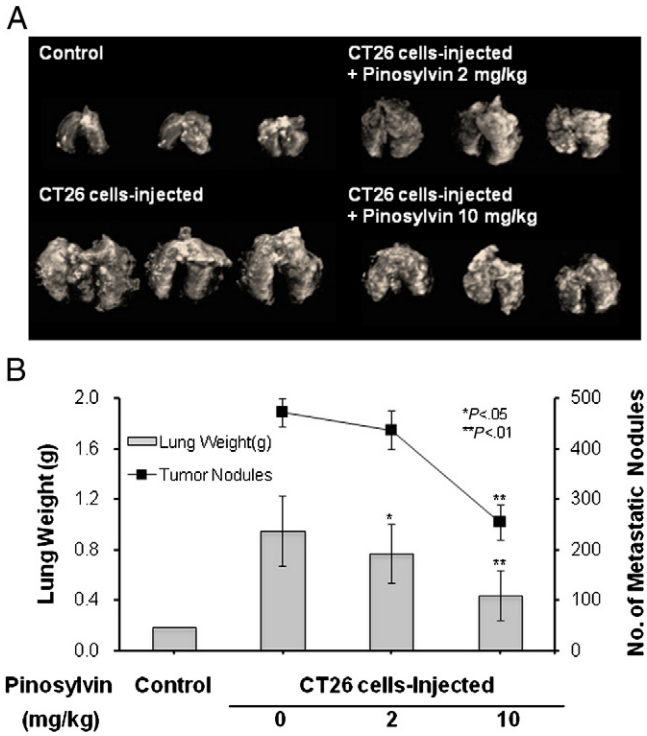

Fig. 4. Inhibitory effects of pinosylvin on the formation of tumor nodules and the increase of lung weight in CT26-induced lung metastasis model. Male Balb/c mice (n=11) undergoing intravenous injection of 1×10^5 CT26 cells via tail vein were treated with vehicle or pinosylvin (2 mg/kg, 10 mg/kg) for 10 consecutive days. On day 15, the mice were sacrificed and analyzed for changes in lung weight and the number of tumor nodules. The numbers of pulmonary nodule were counted after staining with Bouin's solution. (A) The representative lungs of each group were photographed after sacrifice. (B) The lung weight of each group is presented as a bar of mean ± S.D. The number of pulmonary nodules is also demonstrated with a line graph of mean±S.D. Asterisks denote significant differences of pinosylvin-treated group in comparison with CT26-cells-injected control group (*P<.05, **P<.01).

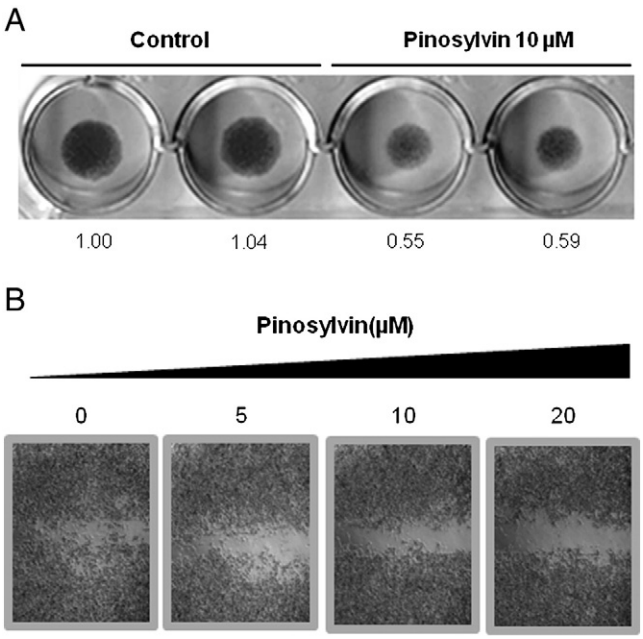

Fig. 3. Effects of pinosylvin on the colony dispersion and wound healing. (A) The migration of HT1080 cells was determined after 48 h of incubation of 10 μM pinosylvin via colony dispersion assay. (B) Confluent cultures of HT1080 cells were wounded with a yellow tip. The cells were incubated with pinosylvin (5, 10 or 20 μM) for 17 h. Data are representative of three independent experiments (n=3).

of pinosylvin compared to the untreated control group during period of experiments.

3.5. Inhibitory effect of pinosylvin on the enzymatic activities of MMPs and COX-2 expression in pulmonary metastasis model

The enzymatic activities of MMP-2 and MMP-9 in lung tissues were evaluated by gelatin zymography. Protein lysates from lung tissues containing tumor nodules were resolved on a 0.2% gelatin-containing SDS–polyacrylamide gel. As a result, the activities of pro-MMP-9 and active MMP-9 in the metastatic lung tissues were increased in the CT26-treated control group compared to the normal group. However, the treatment with pinosylvin inhibited the enzymatic activities of MMP-9 evoked by the CT-26 cells in a dose-dependent manner. Compared to MMP-9 activity, the MMP-2 enzymatic activity in CT-26-treated lung tissue was not much increased, and the inhibitory effect of pinosylvin was also relatively low (Fig. 5).

Cyclooxygenase-2 is considered to be up-regulated in metastatic lesions, and overexpression of COX-2 affordably induces metastasis in tumor tissues [27]. Based on these findings, the expression of COX-2 in each group was examined by Western blot analysis and RT-PCR. Lung tissues from the CT26-treated control group exhibited an increase of the protein and mRNA expressions of COX-2 compared to normal control. However, the pinosylvin-treated group exhibited a suppression of the protein (Fig. 6A) and mRNA expression of COX-2 in a dose-dependent manner (Fig. 6B). In addition, the phosphorylation

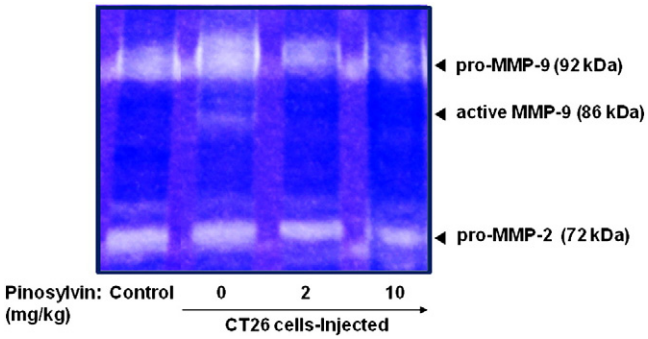

Fig. 5. Inhibitory effect of pinosylvin on the MMP-2 and MMP-9 enzymatic activities in the CT26-induced pulmonary metastasis model. The protein lysates from the median lungs of each group were subjected to gelatin zymography. Proteolytic activities of MMP-2 and MMP-9 are demonstrated as a clear band.

of p44/42 (ERK1/2) that is thought to be one of the upstream signal proteins of COX-2 as well as MMP-9 was also analyzed. Pinosylvin resulted in the suppression of ERK1/2 by phosphorylation at both Thr202 and Tyr204 in lung tissues without changes in total amount of ERK1/2. In addition, the phosphorylation of Akt at Tyr308, the active form of Akt, was also down-regulated by the treatment of pinosylvin (Fig. 6A).

4. Discussion

Metastasis is one of the main factors of mortality in cancer patients. In the process of metastasis of cancer cells, the degradation of basement membrane is an obstacle barrier, and the proteolytic enzymes such as MMPs are also involved. Matrix metalloproteinases are crucial factors for the destruction of ECM in invasion and metastasis [3], and the overexpression and aberrant activation of MMPs are also reported in various tumors [27–29]. Among MMPs, MMP-2 and MMP-9 with gelatinase activities are known to be closely involved in the invasion and metastasis [3]. Indeed, several cancer chemopreventive agents including curcumin and polyphenolic compounds exhibited an antimetastatic potential with inhibitory effects on a pulmonary metastasis of B16F10 in mice [30]. In addition, a chemopreventive agent, resveratrol, was also reported to inhibit the MMP-2 and -9 activities [31–33]. Although pinosylvin has been reported to have a chemopreventive potential to regulate the COX-2 and iNOS expressions [20,34] that are highly correlated with inflammation and metastasis processes, the effect of pinosylvin on metastasis has not been elucidated yet. The present study, therefore, was designed to investigate the effect of pinosylvin on metastatic processes.

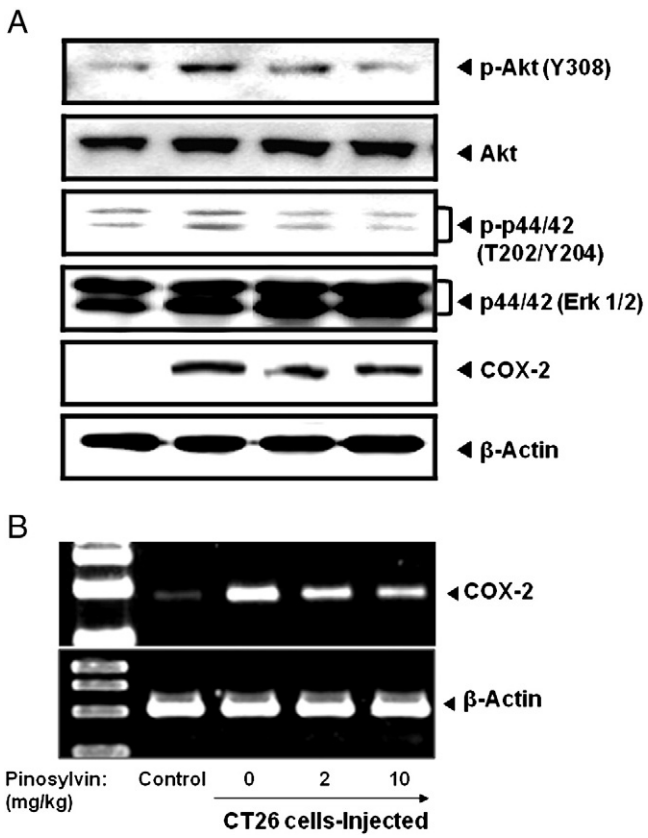

Fig. 6. Suppression of pinosylvin on the expressions of phosphorylation of Akt, p44/42 (Erk1/2) and COX-2 in the CT26-induced pulmonary metastasis model. (A) Protein and (B) RNA were extracted from the lungs as described in Materials and methods and applied to Western blot analysis and RT-PCR, respectively. Data are representative of three independent experiments (n=3).

Primarily, employing the short-term experiments of the spontaneous pulmonary metastasis model in mice, the effect of pinosylvin on the metastasis was evaluated *in vivo*. Murine colon CT26 cancer cells were injected into Balb/c mice intravenously, and the formation of tumors in the lung tissues was measured with the treatment of test compounds for 2 weeks. As a result, the injection of CT26 cells induced the formation of tumor nodules in lung and increased the mass of lung tissues, but the treatment of pinosylvin significantly alleviated the formation of tumor nodules and increase of lung mass in a dose-dependent manner. This result suggests that pinosylvin might have a potential to inhibit the metastatic activity of tumor cells *in vivo*.

To further elucidate the underlying mechanisms of actions of the antimetastatic activity of pinosylvin in an animal model, the lung tissues were analyzed. The metastasis biomarkers MMP-9 and COX-2 were highly up-regulated by the CT26 cell injection in lung tumor nodules, but the treatment of pinosylvin suppressed their expressions in terms of mRNA or protein levels. The positive correlation between COX-2 and metastasis has been reported in many studies, in which suppression of the metastasis as well as the expression of COX-2 itself by selective COX-2 inhibitors including celecoxib, etodolac and NS-398 was observed in various malignant lesions [9,35,36]. In addition, we already reported that pinosylvin and its derivatives suppressed COX-2 expression and prostaglandin E_2 production in lipopolysaccharide-induced mouse macrophage RAW264.7 cells [20]. Therefore, it is assumed that the antimetastatic effects of pinosylvin might be in part due to the suppression of the COX-2 expression as well as MMPs.

Moreover, on the basis of this information, further studies were performed to examine the effect of pinosylvin on the ERK1/2 activation, the upstream signaling molecules of MMP-9 and COX-2. Pinosylvin caused the down-regulation of active ERK1/2 phosphorylation at Tyr202 and Tyr204 residues. It was reported that ERK1/2, a member of MAPKs, stimulates the expression of MMPs [4], and an inhibitor of ERK1 reduces the *in vivo* invasiveness of head and neck squamous cell carcinoma [37]. In fact, in HT1080 cells, activated Ras contributes to the transformed phenotype of HT1080 cells by activating signal transduction molecules including Raf, MEK and ERK1/2, and also several MMPs are overexpressed and activated in these cells [38–40]. These factors might affect the high potential metastatic characteristics of HT1080 cells. Therefore, it is suggested that the inhibitory effects of pinosylvin on tumor metastasis are in part due to the ERK1/2-mediated down-regulation of COX-2 and MMP-9 expression. It has been reported that Akt significantly enhanced invasiveness and generation of MMP-9 in HT1080 cells [41]. Therefore, the down-regulation of the protein expression of pro-MMP-9 might be caused by the inhibitory effect of pinosylvin on the phosphorylation of Akt.

The involvement of MMPs in the antimetastatic effect of pinosylvin showed that pinosylvin down-regulated the expressions of MMP-2, MMP-9 and MT1-MMP in HT1080 fibrosarcoma cell cultures and MMP-2 and MMP-9 in pulmonary metastatic animal model. These results further confirmed the antimetastatic activity of pinosylvin via the modulation of MMPs as major biomarkers of invasion and metastasis. However, one interesting finding was the suppression of TIMP-1, which is known as an inhibitor of MMP-9 by pinosylvin. The one possible reason for this phenomenon is that the transcription of both MMP-9 and TIMP-1 can be induced by the common transcription factors including AP-1 and NF-κB. In addition, emerging body of evidence suggests TIMP-1 plays other roles in cancer, distinct from the MMP-9 inhibitory activity, and it has been reported that the up-regulation of TIMP-1 in several human cancers exhibited negative prognosis [42]. The hypothesis that TIMP-1 might be positively related to cell survival via the interaction with CD63/integrin β1 complex was also demonstrated in the recent study [43]. Therefore, the inhibition of TIMP-1 by pinosylvin might have a positive effect to inhibit cancer cell survival and metastasis. On the other hand, to elucidate the specific mechanisms of pinosylvin, it is necessary to evaluate the effect of pinosylvin on MMPs induced by specific reagents including TPA or TNF-α. Further study also revealed that the migration of cancer cells was inhibited by the treatment of pinosylvin via the inhibition of wound healing and colony dispersion assay *in vitro*.

In addition, although pinosylvin is a constituent of pine leaves, when considering the clinical relevant dose for activity, as issued by resveratrol, the dietary consumption of natural crude source itself as a tea or other forms may not be sufficient because of little abundance of pinosylvin in pine leaves. With a simple dose translation formulation from the animal data as suggested by Reagan-Shaw et al. [44], the antimetastatic activity of pinosylvin with the dose of 10 mg/kg in mouse might be equivalent to approximately 50-mg dose of pinosylvin for an adult person. As stated previously, although this dose may not be achievable by the consumption of natural sources, the dose (50 mg) or higher might be readily supplemented as tablet or capsule form for general consumption of pinosylvin.

Taken together, pinosylvin has a potential to possess antimetastatic activity *in vivo*, and its effects could be related with the suppression of MMPs. This result provides the additional mechanisms of actions of pinosylvin as a chemopreventive or anticancer agent.

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