Angelica sinensis suppresses human lung adenocarcinoma A549 cell metastasis by regulating MMPs/TIMPs and TGF-β1

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Abstract. In this study we investigated the potential effects of *Angelica sinensis* on the growth and metastasis in human lung adenocarcinoma A549 cells. *In vitro* the Cck-8 assays showed that *Angelica sinensis* had weak antiproliferative effect on A549 cells only at high concentration. The cell adhesion assay showed that *Angelica sinensis* decreased the adhesive ability of A549 cells in a dose- and time-dependent manner. Transwell invasion and migration assay showed that *Angelica sinensis* reduced the invasive and migratory abilities of A549 cells in a dose-dependent manner. *In vivo* the animal experiments showed that *Angelica sinensis* suppressed lung metastasis of nude mice at high concentration. Then, we attempted to clarify the mechanisms of anti-metastatic activities of *Angelica sinensis*. The results showed *Angelica sinensis* inhibited the enzymatic activity of matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9), it involved the down-regulation of the expressions of MMP-2 and MMP-9 at both the protein and mRNA levels, which may be associated with *Angelica sinensis* suppressing the expression of TGF-β1. It also involved the increase of the tissue inhibitors of metalloproteinases TIMP-2, but TIMP-1 decreased upon incubation of A549 cells with *Angelica sinensis*. The results suggest that *Angelica sinensis* might exert anti-growth and anti-metastasis activity against lung cancer cells through the decrease of MMP-2, MMP-9, TGF-β1 and TIMP-1 and increase of TIMP-2.

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Abbreviations: MMP-2, matrix metalloproteinase-2; MMP-9, matrix metalloproteinase-9; TIMP-1, tissue inhibitors of metalloproteinases-1; TIMP-2, tissue inhibitors of metalloproteinases-2; NSCLC, non-small cell lung carcinoma; ECM, extracellular matrix; MMPs, matrix metalloproteinases; TIMPs, tissue inhibitor metalloproteinases

Key words: Angelica sinensis, metastasis, MMP-2, MMP-9, transforming growth factor β

Introduction

Non-small cell lung carcinoma (NSCLC) is currently one of the leading causes of death in China, its high mortality is attributed to early metastasis. About 70-90% patients with lung cancer need radiation therapy, and therefore, radiation lung injury is the most common complication (1). It limits the increasing of radiation dose and influences the life quality of patients or even threatens their life. Many traditional Chinese medicines have great efficacy in radiation lung injury. The root of *Angelica sinensis*, also known as 'Danggui' was a popular herbal medicine and widely used for many diseases in China. It has been used for more than 10 years in our hospital to treat lung fibrosis and shows good curative effect in radiation pulmonary fibrosis (2-4).

Angelica sinensis belongs to 'activating blood and dissolving stasis' kind of traditional Chinese medicine, and some researchers think this kind of medicine can promote tumor metastasis. Li *et al* first reported *Salvia miltiorrhiza Bunge* and *Radix Paeoniae Rubra* such as 'activating blood and dissolving stasis' traditional Chinese medicine can promote tumor metastasis, we pay high attention to it (5). Han *et al* found the metastasis rate of nasopharyngeal carcinoma increased to 2.67 times compare with the control after using 'activating blood and dissolving stasis' kind of traditional Chinese medicine (6). But also, there is research with the opposite conclusion (7). Therefore, the use of *Angelica sinensis* needs to be clarified to confirm whether it promotes or inhibits metastasis of lung cancer cells. Adenocarcinoma of the lung is more metastatic compared to squamous carcinoma. So, we chose the high metastasis ability human lung adenocarcinoma cell line A549 cells as our research object.

Metastasis of cancer cells involves multistep processes, including changed adhesion ability between the cells and the extracellular matrix (ECM) and damaged intercellular interaction. The degradation of basal membrane and ECM of primary tumor are crucial steps for tumor invasion and metastasis. Matrix metalloproteinase (MMP) family of human zinc-dependent peptidases is responsible for degradation of the ECM (8). Among the many MMPs that have been identified, MMP-2 (gelatinase-A) and MMP-9 (gelatinase-B) are thought to be key enzymes because they efficiently degrade native collagen types IV, which are the main component of

ECM (9-12). Many studies have shown that overexpression of MMPs is correlated with the progression of lung cancer, which contributes to tumor invasion, metastasis and angiogenesis (13). The expression of either MMP-9 or MMP-2 confers a worse prognosis in early stage adenocarcinoma of the lung (14).

All members of the MMPs family can be regulated by their endogenous inhibitors, the tissue inhibitor metalloproteinases (TIMPs), which prevent the degradation of ECM by binding MMP non-covalently in a 1:1 stoichiometric complex (15-17). Therefore, MMP activity is determined by the balance of MMP and TIMP expression. At least four TIMPs (TIMP-1, TIMP-2, TIMP-3 and TIMP-4) have been identified (18). The enzymatic activity of MMP-2 and MMP-9 is blocked by TIMP-2 and TIMP-1, respectively (19,20).

MMP-2 and MMP-9 can be up-regulated by TGF-β (21). High TGF-β activity is associated with highly aggressive and proliferative of gliomas and a poor prognosis in patients (22). TGF-β is also one of the critical regulators in the inflammatory reaction that orchestrates the tumor microenvironment. The studies before showed *Angelica sinensis* indicated its clinical efficacy in treating radiation-induced pneumonitis by down-regulation of TNF-α and TGF-β1 (23,24). So, *Angelica sinensis* have potentially the ability to inhibit MMP-2 and MMP-9 by down-regulation of TGF-β1.

In order to define in more detail the role of *Angelica sinensis* in the lung cancer metastatic process, A549 lung cancer cell line was treated with *Angelica sinensis* and examined for the following: i) the relationship between the *Angelica sinensis* and the cell proliferation, adhesion, migration, and invasion ability of A549 lung cancer cells to know whether *Angelica sinensis* inhibited tumor growth and metastasis. ii) the impact of *Angelica sinensis* on MMP-2 and MM-9 regulation including of enzymatic activity, transcriptional, post-transcriptional, TIMPs and TGF-β1, to investigate the molecular mechanisms of *Angelica sinensis*-mediated malignant progression of human lung carcinoma.

Materials and methods

Cell line and culture conditions. The A549 human lung adenocarcinoma cells were obtained from Typical Species Preservation Center of Wuhan University (Wuhan, China) and cultured in RPMI-1640 (Gibco/BRL) media with 10% fetal calf serum (Hangzhou Evergreen Biological Corp., China) in the presence of 5% CO_2. *Angelica sinensis* injection (25%): produced by Zhongnan Hospital of Wuhan University Pharmacies, batch number 960812. The A549 cells were treated with various concentrations (0, 2.5, 10 and 25 mg/ml) *Angelica sinensis* for 24 h before investigation *in vitro*.

Cell viability assay. The effect of *Angelica sinensis* on A549 cell growth was examined by the the Cell Counting Kit-8 (CCK-8) (Dojindo Molecular Technologies, Inc., Gaithersburg, MD) assay following the manufacturer's protocol. Cells were seeded at a density of $2x10^4$ cells/well in a 96-well plate for 24 h. Then, the cells were treated with *Angelica sinensis* at various concentrations (0, 2.5, 10 and 25 mg/ml) for various periods of time (12, 24, 48 and 76 h). Each concentration was repeated six times. After the exposure period, 10 μl of WST-8 was added to each well and allowed to incubate for 3 h. Finally, the absorbance of OD at 450 nm was detected and recorded with microplate reader (Rayto, RT-6000, USA). Each point was expressed at the mean ± SD from three different experiments.

Cell adhesion assay. The MTT assay was performed to calculate the number of attached cells. Each well of a 96-well plate was coated with 0.1% fibronectin (Beijing University Biology Center, China). The plates were washed and incubated with 2% bovine serum albumin for 2 h. The A549 cells ($4x10^4$) were treated with various concentrations *Angelica sinensis* (0, 2.5, 10 and 25 mg/ml) for 24 h at 37˚C, then cells were added to each well and incubated at 0.5-, 1- and 2-h intervals at 37˚C. Unattached cells were removed by washing with PBS. MTT was added to each well and incubated for 4 h at 37˚C. Formazen crystals were dissolved by addition of DMSO solution. The absorbance of each well was determined using the microplate reader (Rayto, RT-6000) at 570 nM.

Cell invasion and migration assays. The ability of A549 cells pass through matrigel-coated filters was examined by the tranwell invasion assay. The assay was measured using transwell chambers (Corning Costar, MA) with 8-μm pore size polycarbonate filter according to the manufacturer's. First, Matrigel (BD Biosciences, Bedford, MA) was applied to the top side of the polycarbonate filter. A549 cells were resuspended in serum-free medium after treating with various concentrations of *Angelica sinensis* (0, 2.5, 10 and 25 mg/ml) for 24 h. Medium containing 10% FBS was applied to the lower chamber as chemoattractant, the cells ($2x10^4$ cells/well) were seeded on the upper chamber and incubated for 16 h at 37˚C. At the end of incubation, the cells in the upper surface of the membrane were removed with a cotton swab and cells invaded across the matrigel to the lower surface of the membrane were fixed with methanol and stained with 0.1% Crystal violet. The cells that invaded the lower surface of the filter were quantified under a microscope (x20). Each experiment was carried out in triplicate. The migration assay was performed as described for the invasion assay, but without the coating of matrigel.

Animal assay. Nude mice (8-10 weeks old) were purchased from the Hubei Provincial Disease Control and Prevention Center and maintained in a germ-free environment in the animal facility. In the model, the highly metastatic A549 cells ($2x10^5$ cells) injected into the tail vein of nude mice and divided into four groups (n=8 each). The mice were treated with PBS (control group) or *Angelica sinensis* (0.5, 1 and 5 mg/g) p.i. (intraperitoneal injection) three times a week for 14 days. Then, the mice were sacrificed, lungs were removed, weighed and mouse lungs were injected intratracheally with India ink and fixed in Fekete's solution, the metastatic tumors in each lung were counted under a dissecting microscope. The experiments were performed three times. The experimental protocols were reviewed and approved by the Animal Research Committee of Zhongnan Hospital of Wuhan University.

Gelatin zymography. The enzymatic activity of MMP-2 and MMP-9 in the conditioned medium of A549 cells was determined by gelatin zymography. The conditioned medium was

Table I. The primers used for quantitative real-time PCR.

Gene	Primer sequences 5'→3'
β-actin	Forward: GTCCACCGCAAATGCTTCTA
	Reverse: TGCTGTCACCTTCACCGTTC
TGF-β1	Forward: GCAGCTCCTTTAGGTTCTT
	Reverse: TTCCTGGGCTGGGCTGGACC
MMP-2	Forward: AGTGACGGAAAGATGTGGTGTG
	Reverse: CTTGGTGTAGGTGTAAATGGGTG
MMP-9	Forward: TCCCTGGAGACCTGAGAACC
	Reverse: CGGCAAGTCTTCCGAGTAGTTT
TIMP-1	Forward: ACTTCCACAGGTCCCACAAC
	Reverse: AGCCACGAAACTGCAGGTAG
TIMP-2	Forward: CCGCTCAAATACCTTCACAAT
	Reverse: TTACGGCAGCAAGTCCAATA

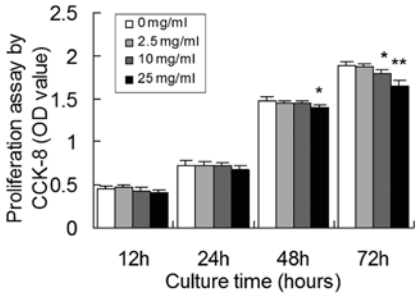

Figure 1. Effect of *Angelica sinensis* on the proliferation of A549 cells. The cells ($2x10^4$ cells/ml) were treated with various concentrations (0, 2.5, 5 and 10 mg/ml) of *Angelica sinensis* for 12, 24, 48 and 72 h. Cell viability was determined by CCK-8 assay. The surviving cell number was directly proportional to that of formazan, which was measured with microplate reader at 450 nm. Each point was expressed at the mean ± SD from six different experiments. (*P<0.05, **P<0.001 compared to the untreated control at the same periods of time).

resolved by 10% SDS-PAGE containing 0.1% gelatin after A549 cells were treated with various concentrations (0, 2.5, 10 and 25 mg/ml) *Angelica sinensis* for 24 h. After electrophoresis, gels were washed with distilled water containing 2.5% Triton X-100, then incubated in reaction buffer (50 mmol/l Tris-HCl, 5 mmol/ $CaCl_2$, 1 μmol/l $ZnCl_2$ and 0. 02% Brij-35) for 48 h at 37˚C. Then the gels were stained with Coomassie brilliant blue R-250. Non-staining bands representing the levels of the latent form of MMP-2 and MMP-9 were quantified by densitometer measurement using a digital imaging analysis system (GeneGenius, Syngene, UK).

SYBR-Green real-time quantitative PCR. We examined the effects of *Angelica sinensis* on expression of TGF-β1, MMP and TIMP mRNA in A549 cells using SYBR-Green real-time quantitative PCR. A549 cells were treated with various concentrations (0, 2.5, 10 and 25 mg/ml) of *Angelica sinensis* for 24 h. Total RNA was extracted from the cells using an TRIzol Reagent (Toyobo, Japan) according to the manufacturer's instructions. The complementary DNA (cDNA) was produced from the extracted total RNA using a Reverse Transcriptase kit (Toyobo). Primer sequences are shown in Table I. Quantitative real-time PCR was performed using SYBR-Green PCR master mix (Toyobo) on the sequence detection system (Stratagene Mx3000p, USA), and the fluorescence was collected thrice during each cycle as described below. PCR was performed at 95˚C for 15 sec, 58˚C for 15 sec, and 72˚C for 15 sec for 40 cycles. All the samples were run in triplicates, β-actin was used as an internal control in each run. Results of the real-time PCR were expressed as C_t, C_t is the cycle number at which amplification fluorescence reaches a value above a preset threshold. The gene expression was measured by relative quantitative $2^{-\Delta\Delta Ct}$ method (25). ΔC_t = C_t value of target-C_t value of β-actin; $\Delta\Delta C_t$ = ΔC_t value of target group-ΔC_t value of untreated control group, the expression of the untreated control was 1, $2^{-\Delta\Delta Ct}$ value is the section of the treated group for control group.

Western blot analysis. A549 lung cancer cells were treated with different concentrations of *Angelica sinensis* for 24 h, the cells were harvested, and cellular proteins were extracted with lysis buffer. Total protein was separated by 12% SDS-PAGE and transferred to nitrocellulose membranes. After blocking with 5% non-fat dry milk, membranes were incubated with the primary antibodies of TGF-β1, MMP-2 and MMP-9 (Biomeda Corp., Foster City, CA) overnight at 4˚C. The membranes were incubated with the appropriate peroxidase-conjugated secondary antibodies, and detected with ECL reagent. Protein levels were quantitated by a digital imaging analysis system (GeneGenius).

ELISA. TGF-β1, MMP-2, MMP-9, TIMP-1 and TIMP-2 in conditioned medium was measured by ELISA kit (R&D Systems) following the manufacturer's protocol. A549 cells were seeded at a density of $2x10^4$ cells/well in a 96-well plate for 24 h, cells were treated with various concentrations (0, 2.5, 10 and 25 mg/ml) *Angelica sinensis* for 24 h. The culture media were collected at the end of incubation. The samples were added to each well of ELISA kit and incubated for 2 h at 37˚C. The detection antibody 50 μl was added and incubated for 1 h at 37˚C. The working dilution of streptavidin 100 μl was added to each well and incubated for 60 min at 37˚C. The substrate solution 50 μl was added and incubated for 5-10 min at 37˚C. After the addition of stop solution, the optical density of each well was measured immediately with a microplate reader set to 492 nm.

Statistical analysis. Values are expressed as means ± SD of three independent experiments and a two-sample Student's t-test was performed to compare the treated groups with untreated control. Values of P<0.05 were regarded as statistically significant.

Results

Effects of Angelica sinensis on the viability of A549 cells. In this study, we examined the effects of *Angelica sinensis* on the proliferation of A549 cells using the CCK-8 assay. The A549 cells were treated with *Angelica sinensis* at various concentrations (0, 2.5, 10 and 25 mg/ml) for 12, 24, 48 and 72 h. Comparing to that of control (untreated

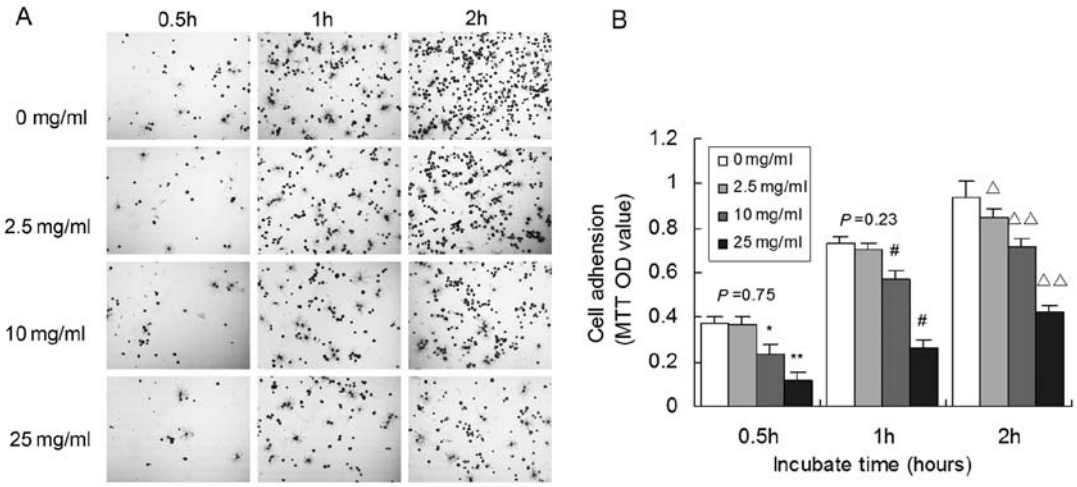

Figure 2. Inhibitory effects of *Angelica sinensis* on the cell-fibronectin adhesion of A549 cells. The cells ($4x10^4$) were added to a 96-well plate coated with 0.1% fibronectin before the cells were treated with various concentrations of *Angelica sinensis* (0, 2.5, 10 and 25 mg/ml) for 24 h, and the cells were incubated at 0.5-h, 1-h and 2-h intervals. (A) The cells adhered to the well (x20 fold magnification). (B) The number of attached cells were calculated by the MTT assay. Result showed *Angelica sinensis* inhibit the cell-fibronectin adhesion. Values are expressed as the mean ± SD of three independent experiments. (*P<0.05, **P<0.001 compared to the untreated control at 0.5 h; $^\#$P<0.001 compared to the untreated control at 1 h; $^\triangle$P<0.05, $^{\triangle\triangle}$P<0.001 compared to the untreated control at 2 h.

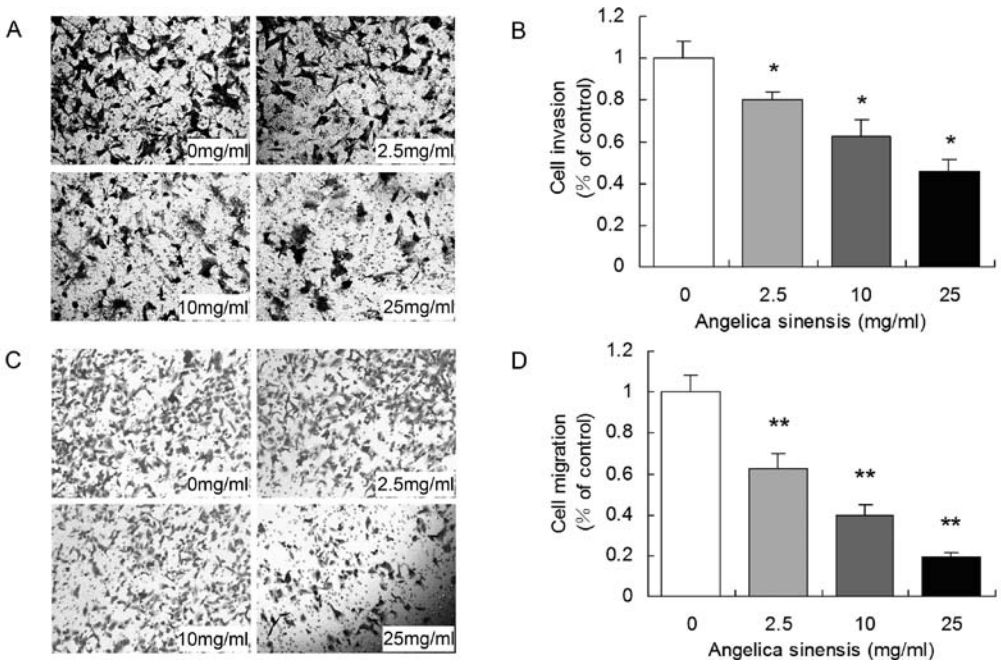

Figure 3. Inhibitory effects of *Angelica sinensis* on the invasion and migration of A549 cells. (A and C) Images of the invasion (200-fold magnification) and migration (100-fold magnification) were taken under a microscope. (B and D) The number of transwell invasion cells and migration cells were calculated, untreated control were taken as 100%. The invasive and motility potentials of A549 cells were significantly inhibited by *Angelica sinensis*. Values are expressed as mean ± SD of three independent experiments. (*P<0.05, **P<0.001 compared to the untreated control).

with *Angelica sinensis*), the viability of A549 cells was not significantly affected at the dosage 2.5 mg/ml (Fig. 1). At the period of 48 h, we observed weak growth inhibition even at the maximum concentration of 25 mg/ml, the values were not much greater than those observed for the untreated control. It appears that *Angelica sinensis* requires >25 mg/ml and 48 h to affect A549 cell proliferation. These results imply that multistep molecular events are necessary for function of *Angelica sinensis* to switch the A549 cells from a proliferative state to an inhibited state of cell growth.

Angelica sinensis inhibits adhesion, invasion and migration of A549 cells. Cell-fibronectin adhesion, cell invasion and cell motility are important for cancer cell metastasis. To examine the potential anti-metastasis effects of *Angelica sinensis* on A549 cells, we examined the effects of *Angelica sinensis* on the cell-fibronectin adhesion using the MTT assay. The results showed that at the dosage of 2.5 mg/ml, *Angelica sinensis* has no obvious anti-adhesion effect to A549 cells at 0.5-h and 1-h intervals. On the contrary, at the dosage 10 and 25 mg/ml, we observed a significant anti-adhesion effect at all intervals. It

Table II. Effects of *Angelica sinensis* on tumor metastasis to the lung in a mouse model.

Groups	No. of mice	No. of metastatic nodules	Weight of lungs (mg)
Control (mg/g)	8	124±27	220.8±9.3
0.5	8	117±30	212.3±5.6
1	8	82±17[a]	197.1±6.0[b]
5	8	58±21[b]	176.3±6.7[b]

Effect of *Angelica sinensis* on lung metastasis of nude mice was determined as described in the text. The mice were sacrificed 14 days after cell injection, and the number of lung metastasis and the whole lung weight were measured. Lung metastatic nodules >0.5 mm in diameter were counted. The results indicate the mean ± SD from three independent preparations. Statistically significant value compared with untreated control ([a]P<0.05, [b]P<0.001).

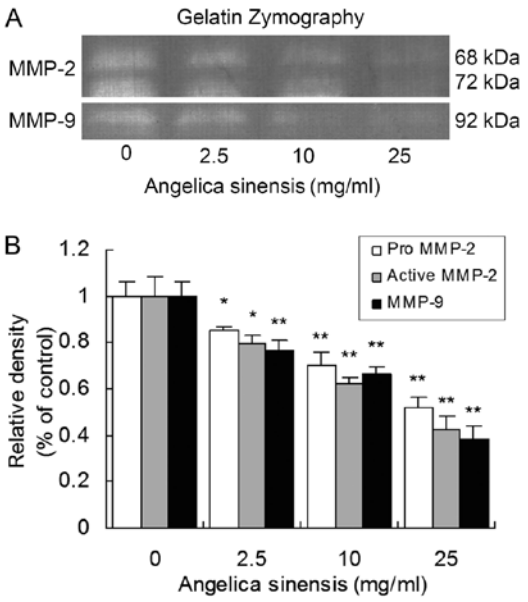

Figure 4. *Angelica sinensis* inhibits MMP-2 and MM-9 activity. MMP-2 and MMP-9 activities were determined by gelatin zymography. (A) The 68- and 72-kDa bands may correspond to active and latent MMP-2 forms. (B) MMP-2 and MMP-9 activities were quantified by densitometric analysis. *Angelica sinensis* reduced MMP-2 and MMP-9 activity in a dose-dependent manner. Results are the mean ± SD from three independent preparations (*P<0.05, **P<0.01 compared to the untreated control).

appears that *Angelica sinensis* requires >2.5 mg/ml to significantly affect A549 cell adhesion (Fig. 2). We examined the effects of *Angelica sinensis* on invasion and migration of A549 cells using a cell invasion and migration assay with the transwell chamber. The results showed *Angelica sinensis* induced a dose-dependent decrease in invasion and migration with an increasing concentration of *Angelica sinensis*. The invasive activities of A549 cells were significantly (P<0.05) reduced to 63% by treatment of 10 mg/ml *Angelica sinensis*, and reduced to 46% by treatment of 25 mg/ml *Angelica sinensis* (Fig. 3A and B). The migrative activities of A549 cells were significantly (P<0.001) reduced to 40% by treatment of 10 mg/ml *Angelica sinensis*, and to 19% of 25 mg/ml *Angelica sinensis* compared to untreated group (Fig. 3C and D). The results demonstrated that *Angelica sinensis* significantly inhibited the invasion and migration of highly metastatic A549 lung cancer cells.

Effects of Angelica sinensis on tumor metastasis to the lung in a mouse model. The studies described above clearly showed that *Angelica sinensis* has a potent ability to inhibit the metastasis of A549 cells *in vitro*. To investigate the role of *Angelica sinensis* in metastasis *in vivo*, we used an animal model to analyze the metastatic potential of A549 cells. In the model, the highly metastatic A549 cells were injected into the tail vein of nude mice, the mice were treated with *Angelica sinensis* (0.5, 1 and 5 mg/g) p.i. three times a week for 14 days. When the mice were treated with 0.5 mg/g *Angelica sinensis* p.i. there was no obvious difference of metastatic colonies and the weights of lung compare with the untreated control (P=0.53, P=0.25). But lung metastasis and weights were significantly decreased when the concentration of *Angelica sinensis* was increased to 5 mg/g suggesting *Angelica sinensis* reduced the metastatic ability only at high concentration. This experiment was performed three times with similar results (Table II).

Angelica sinensis inhibits the MMP-2 and MMP-9 activities of A549 cells. To examine the possible anti-metastatic mechanisms of *Angelica sinensis*, we determined the activity of MMP-2 and MMP-9 in culture media of A549 cells by zymographic analysis. The result showed MMP-2 and MMP-9 activity were suppressed in a dose-dependent manner by treatment of *Angelica sinensis* (0, 2.5, 10 and 25 mg/ml). The active MMP-2 activity was reduced to 62% (P<0.01) by treatment of 10 mg/ml *Angelica sinensis*, and reduced to 42% (P<0.01) by treatment of 25 mg/ml *Angelica sinensis* compared with untreated group. The MMP-9 activity was reduced to 66% (P<0.01) by treatment of 10 mg/ml *Angelica sinensis*, and reduced to 38% (P<0.01) by treatment of 25 mg/ml *Angelica sinensis* compare with untreated group (Fig. 4). These results suggest that the anti-metastatic effect of *Angelica sinensis* is related to inhibition of the enzyme activity of MMP-2 and MMP-9.

Angelica sinensis regulates MMP-2 and MMP-9 levels. To investigate whether the metastasis inhibitory effect of *Angelica sinensis* resulted from the suppression of MMP-2 and MMP-9 expression, MMP-2 and MMP-9 protein and mRNA levels were measured. The protein levels from whole-cell lysates of MMP-2 and MMP-9 were assessed using Western blotting (Fig. 5A and B), and the protein levels in the culture medium was measured by an ELISA (Fig. 5C and D), respectively. The intracellular protein levels of MMP-2 and MMP-9 by Western blotting decreased to 26% (P<0.01) and 44% (P<0.01), and extracellular protein levels of MMP-2 and MMP-9 by an ELISA decreased to 43% (P<0.01) and 37% (P<0.01) after treatment of 25 mg/ml *Angelica sinensis*. Quantitative real-time PCR with SYBR-Green (Fig. 5E) was further employed to analyze the effect of *Angelica sinensis* on the mRNA transcriptional expression of MMP-2 and MMP-9. The mRNA expression of MMP-2 and MMP-9 also decreased in a dose-dependent manner. The changes in the protein levels

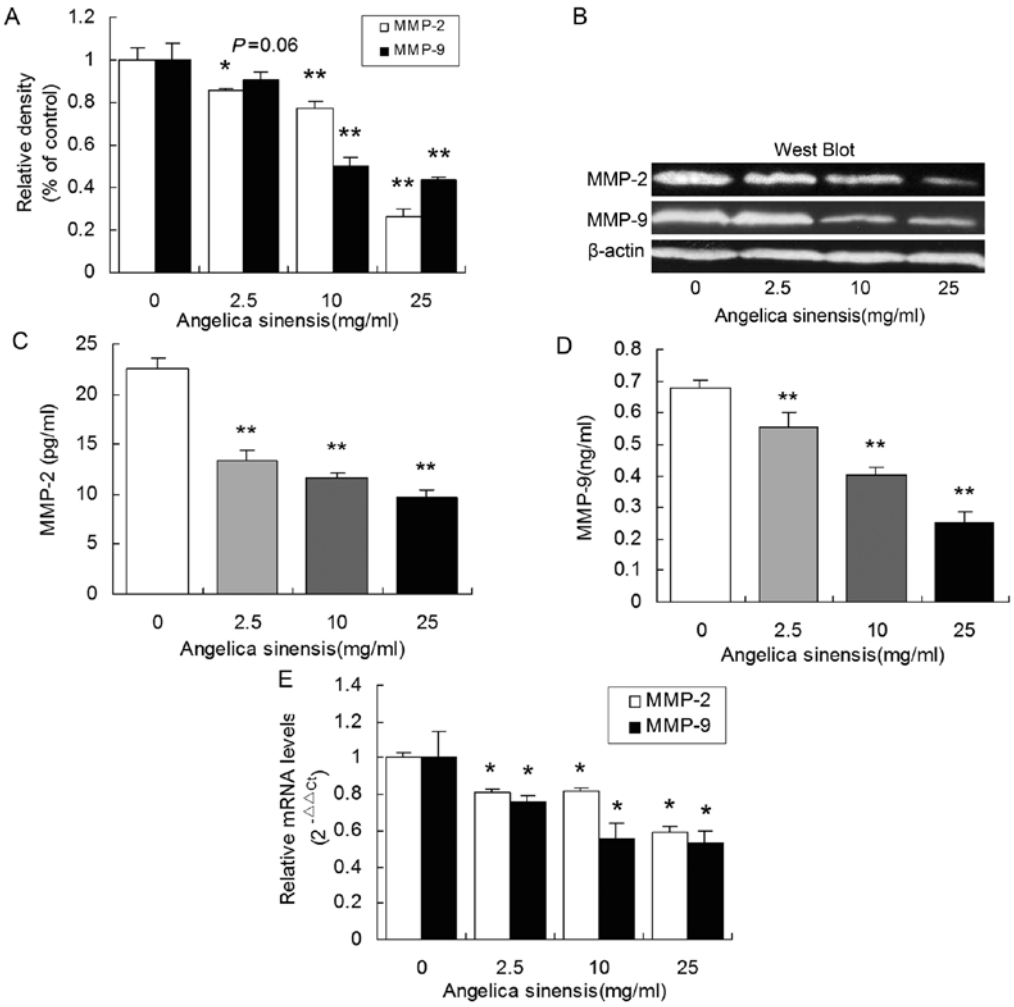

Figure 5. A*ngelica sinensis* inhibits MMP-2 and MMP-9 protein and mRNA levels. (A) MMP-2 and MMP-9 protein levels were quantified by densitometric analysis. (B) Western blot analysis of MMP-2 and MMP-9. (C and D) MMP-2 and MMP-9 concentration in the culture medium was measured by ELISA. (E) Relative mRNA levels of MMP-2 and MMP-9 were quantitated by real-time SYBR-Green PCR and the $2^{-\Delta\Delta Ct}$ method. The expression of the untreated control was 1, $2^{-\Delta\Delta Ct}$ value is the section of the treated group for control group. *Angelica sinensis* reduced MMP-2 and MMP-9 protein and mRNA expression. The results indicate the mean ± SD from three independent preparations. Statistically significant value compared to untreated control ($^{*}P<0.05$, $^{**}P<0.01$ compared to untreated control).

of MMP-2 and MMP-9 coincided with their mRNA levels, indicating that *Angelica sinensis* might regulate MMP-2 and MMP-9 expressions at the transcriptional level.

Angelica sinensis increase levels of TIMP-2 but decrease levels of TIMP-1. Activity of MMP-2 and MMP-9 are significantly related to their inhibition of TIMPs, especially TIMP-1 and TIMP-2. In order to further relate the regulatory effects of *Angelica sinensis* on inhibitor of MMP-2 and MMP-9, the expression of TIMP-1 and TIMP-2 mRNA and protein levels were measured (Fig. 6). The result showed *Angelica sinensis* obviously increased mRNA and protein levels of TIMP-2, the mRNA expression of TIMP-2 increased to 2.4-fold even at the lowest dose 2.5 mg/ml, and protein levels of TIMP-2 increased to 1.7-fold by treatment of 2.5 mg/ml *Angelica sinensis*. The increasing secretion of TIMP-2 resulted in the inhibition of MMP-2 and MMP-9 activity as assessed by zymographic analysis (Fig. 5A and B). In contrast to TIMP-2, TIMP-1 decreased significantly in three experiment groups ($P<0.05$). TIMP-1 mRNA reduced to 76% and protein levels of TIMP-1 reduced to 50% by treatment of 25-mg/ml *Angelica sinensis*. These results suggest that *Angelica sinensis* inhibition of the enzyme activity of MMPs (especially MMP-2) is related to the increase of TIMP-2 but not TIMP-1.

Angelica sinensis inhibits the TGF-β1 expression of A549 cells. TGF-β promotes tumor progression through the up-regulation of matrix metalloproteinase MMP-2 and MMP-9. We investigated whether the MMP-2 and MMP-9 inhibitory effect of *Angelica sinensis* resulted from the suppression of TGF-β1 expression, TGF-β1 mRNA and protein levels were measured. The protein levels from whole-cell lysates of TGF-β1 were assessed using Western blotting, the protein levels in the culture medium was measured by ELISA. The intracellular and extracellular protein levels of TGF-β1 all decreased in a dose-dependent manner. The mRNA expression of TGF-β1 also decreased in a dose-dependent manner. *Angelica sinensis* inhibited the TGF-β1 expression of A549 cells, the MMP-2 and MMP-9 inhibitory effect of *Angelica sinensis is* probably through regulation of TGF-β1 expression (Fig. 7).

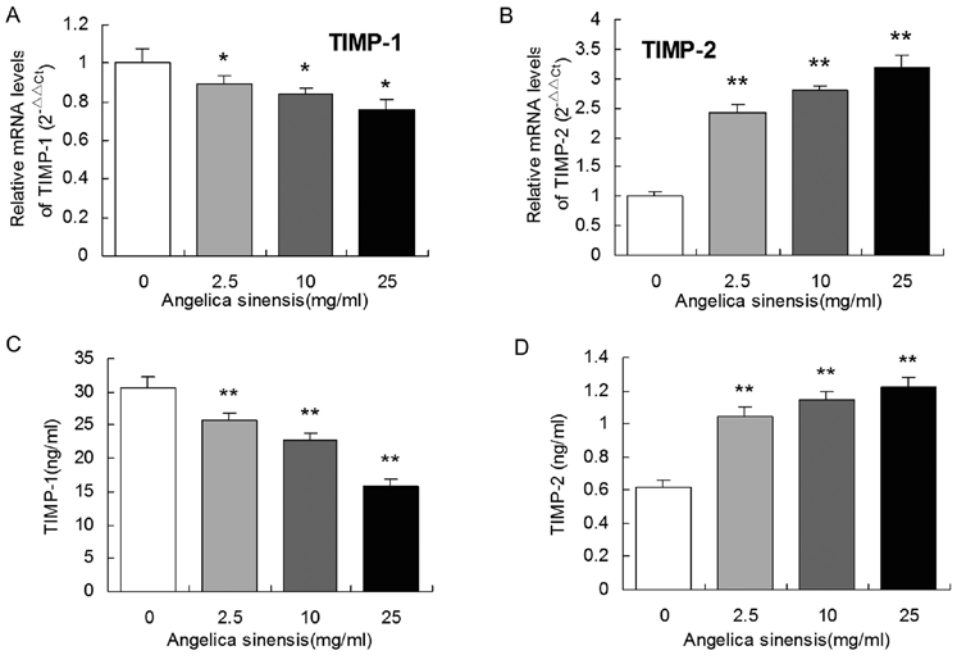

Figure 6. Effect of *Angelica sinensis* on TIMP-1 and TIMP-2 expressions in A549 cells. (A and B) The relative mRNA levels of TIMP-1 and TIMP-2 were quantitated by real-time SYBR-Green PCR and the $2^{-\Delta\Delta Ct}$ method. (C and D) TIMP-1 and TIMP-2 concentration in the culture medium was measured by an ELISA kit as described in Materials and methods. *Angelica sinensis* reduced TIMP-1 but increased TIMP-2 mRNA and protein expression. The results are the mean ± SD from three independent preparations. (*P<0.05, **P<0.001 compared to untreated control).

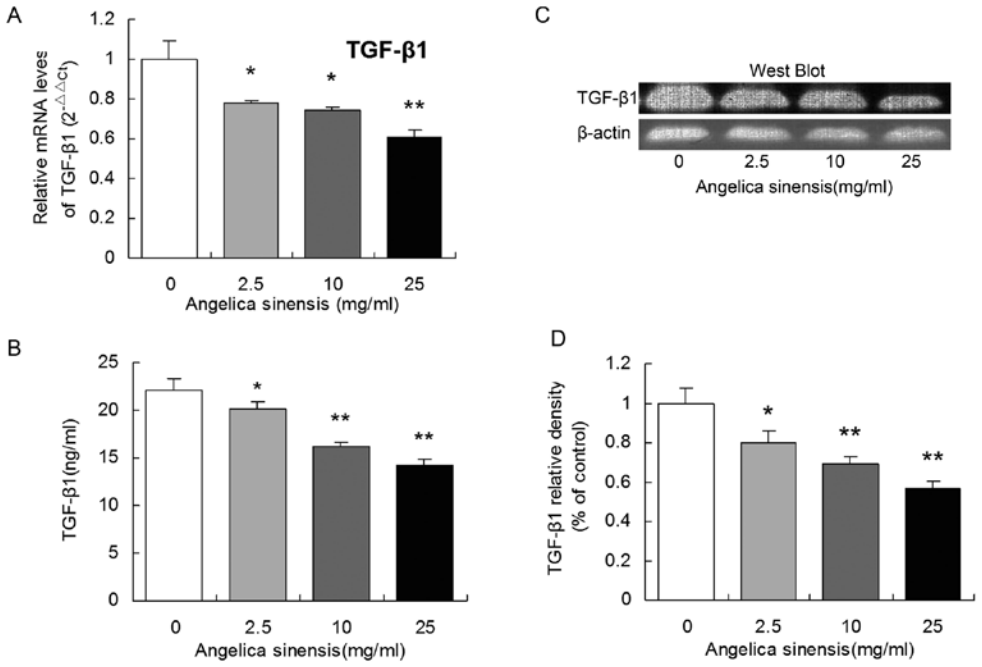

Figure 7. *Angelica sinensis* inhibits TGF-β1 mRNA and protein levels. (A) Relative mRNA levels of TGF-β1 were quantitated by real-time SYBR-Green and the $2^{-\Delta\Delta Ct}$ method. The expression of the untreated control was 1, $2^{-\Delta\Delta Ct}$ value is the section of the treated group for control group. (B) TGF-β1 concentration in the culture medium was measured by an ELISA kit. (C) The protein levels of TGF-β1 from whole-cell lysates were measured by Western blot analysis β-actin served as an internal control. (D) TGF-β1 were quantified by densitometric analysis. *Angelica sinensis* inhibits TGF-β1 mRNA and protein expression. The results are the mean ± SD from three independent preparations. (*P<0.05, **P<0.01 compared to the untreated control).

Discussion

The root of *Angelica sinensis*, also known as 'Danggui' has been a popular herbal medicine and widely used for a long time in China. Various extracts from *Angelica sinensis* were found to have many functions including increasing myocardial ischemia, reduce radiation damages and liver injury, it has also been widely used for treat anemia, thrombosis, and atherosclerosis (26-32).

It has been reported that extracts of *Angelica sinensis* could inhibit the growth of tumor cells (33-40). But, Lau *et al* found the water extract of *Angelica sinensis* stimulated the growth of

MCF-7 cells (41). In this study, *Angelica sinensis* did not show significant cytotoxicity on A549 cells, but when interacted with A549 cells for >48 h at 25 mg/ml, *Angelica sinensis* weakly inhibited the growth of A549 cells. Our results agree with the vast majority. Lau *et al* considered that the water extract of *Angelica sinensis* stimulated the growth of MCF-7 cells, possibly dependent of weak estrogen-agonistic activity. Thus suggesting caution in use of *Angelica sinensis* in women with breast cancer, but it is safe for long-term use in lung cancer patient specially at high concentration.

Tumor metastasis occurs by a series of steps including cell attachment, invasion, and cell proliferation, and is regulated by extremely complicated mechanisms (42). To examine the potential anti-metastasis effects of *Angelica sinensis*, adhesion, invasion and migration assays were performed on A549 cells. *Angelica sinensis* inhibited the adhesion of A549 cells to 0.1% fibronectin, one of the ECM components as well as effectively suppressed the invasion and migration of A549 cells in a concentration-dependent manner suggesting *Angelica sinensis* cannot promote tumor metastasis, but can inhibit tumor cell invasion and metastasis capacity.

Tumor invasion and metastasis require degradation of ECM, and increased expression of proteases associated with these processes. To understand how *Angelica sinensis* inhibited A549 cell metastasis, we focused on *Angelica sinensis* altered proteinases MMP-2 and MMP-9 which are believed to play a critical role in tumor invasiveness and metastasis. We demonstrated that *Angelica sinensis* suppresses the mRNA expression, protein synthesis and activation of MMP-2 and MMP-9 by A549 cells in a dose- and time-dependent manner. We also showed that *Angelica sinensis* inhibited invasion of A549 cells in a dose-dependent manner, indicating that the inhibition of MMP-2 and MMP-9 function resulted in the loss of ability for A549 cells to degrade the component of Matrigel and thus the ability to invade *in vitro*.

Another possible anti-metastatic mechanism of *Angelica sinensis* is through increased expression of TIMP-2. TIMPs are endogenous inhibitors that can block the activities of MMPs. The balance between the levels of activated MMPs and free TIMPs determines overall MMP activity. The overexpression of TIMPs has been demonstrated to reduce experimental metastasis. TIMP-1 and TIMP-2 were known to have a particularly high affinity for MMP-9 and MMP-2, respectively (43). Many studies have found that TIMP-2 overexpression decreased invasion of endothelial and tumor cells both *in vitro* and *in vivo* (44,45). This study showed *Angelica sinensis* obviously increased TIMP-2 expression but decreased TIMP-1 expression, which can inhibit the enzyme activity of MMP-2 and MMP-9.

Angelica sinensis expression inhibits cancer proliferation possibly by inhibiting the TIMP-1. Although it has been reported that TIMP-1 acts as a metastasis suppressor gene, some studies have shown increased expression of TIMP-1 in tumor cells in accordance with the poorer results (46,47). TIMP-1 promotes cell growth (48,49) and inhibits apoptosis (50,51). It was shown recently that TIMP-1 contributes functionally to neoplastic development in a mouse model of epithelial carcinogenesis (52). *Angelica sinensis* inhibits the TGF-β1 expression of A549 cells. TGF-β1 has been shown in several studies of nephropathy to increase the expression of TIMP-1 (53-56), the TIMP-1 inhibitory effect of *Angelica sinensis* is probably through regulation of TGF-β1 expression.

In conclusion, the present study demonstrates that *Angelica sinensis* significantly inhibits the metastasis ability of human lung cancer A549 cells. The proposed anti-invasion mechanisms might be mediated through the inhibition of MMP-2 and MMP-9, TGF-β1 and TIMP-1, as well as enhancement of TIMP-2. Further analysis with semi-quantitative RT-PCR showed that the regulation of MMP-2, MMP-9 and TIMP-2 expressions by *Angelica sinensis* may be on the transcriptional level.

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