

## Cambodian *Phellinus linteus* Inhibits Experimental Metastasis of Melanoma Cells in Mice *via* Regulation of Urokinase Type Plasminogen Activator

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*Phellinus linteus* (PL) is a fungus mainly found in tropical America, Africa and Asian countries including Korea, Japan and China. PL has been traditionally used for the treatment of arthritis, liver damage and cancer. However, little was known on the biological activity and characterization of *Phellinus* species in Cambodia. Thus, in the present study, the anti-metastatic mechanism of aqueous extract of Cambodian *Phellinus linteus* (CPL) was evaluated. Cambodian mushroom was identified as a *Phellinus* species with 99% homology of *Phellinus linteus* by DNA sequence analysis and comparison by the National Center for Biotechnology Information. CPL did not exhibit any significant cytotoxicity against B16BL6 cells, invasive melanoma cells at 1 mg/ml. However, CPL inhibited platelet aggregation induced by B16BL6 cells and also disrupted the adhesion to gelatin and invasion of B16BL6 cells in a concentration dependent manner. Similarly, CPL dose-dependently inhibited the pulmonary metastatic colonies in C57BL/6 mice intravenously injected by B16BL6 cells up to 55.5% at a dose of 50 mg/kg compared with untreated control. CPL also down-regulated the expression of urokinase type plasminogen activator (uPA), one of key proteins associated with invasion and metastasis of tumor cells in a concentration dependent fashion, while CPL didn't significantly affect the expression of matrix metalloproteinase 2 (MMP-2) and tissue inhibitor of metalloproteinase 2 (TIMP-2) by reverse transcriptase–polymerase chain reaction (RT-PCR). Taken together, these findings indicate that Cambodian *Phellinus linteus* may inhibit metastasis at least partly *via* regulation of uPA associated with tumor cell induced platelet aggregation (TCIPA) and also suggest a further study for isolation of active ingredients and the involvement of adhesion molecule signaling pathway.

**Key words** Cambodian *Phellinus linteus*; TCIPA; adhesion; invasion; metastasis; uPA

*Phellinus linteus* (PL), commonly referred as Sangwhang in Korea, is a mushroom belonging to the Hymenochaetales basidiomycetes and also found mainly in tropical America, Africa and Asian countries.<sup>1)</sup> Overall, PL was reported to be a medicinal mushroom with anti-tumor activity,<sup>2,3)</sup> protective effect on liver damage,<sup>4)</sup> immunomodulatory action,<sup>5)</sup> anti-angiogenic and antioxidant activity.<sup>6)</sup> The hot aqueous extract of PL inhibited the growth of sarcoma 180 to about 96.7% of untreated control in ICR mice.<sup>7)</sup> Of different *Phellinus* species, *Phellinus gilvus* and *Phellinus baumii* in Korea inhibited pulmonary inflammation,<sup>8)</sup> *Phellinus rimosus* (BERK) PILAT showed antitumor activity<sup>9)</sup> and anti-oxidant and anti-hepatotoxic activities,<sup>10)</sup> and *Phellinus igniarius* exerted anti-mutagenicity.<sup>11)</sup> Recently the mushroom cultivated in Cambodia with the shape of *Phellinus linteus* has been used for health and prevention of diseases as a remedy of folk. However, little was known on the characterization and biological response of the mushroom. Thus, with aqueous extract of Cambodian mushroom, the characterization of species by DNA analysis and anti-metastatic mechanism were evaluated *in vitro* and *in vivo*.

### MATERIALS AND METHODS

**Reagents** Minimum essential medium (MEM), fetal bovine serum, antibiotic–antimycotic, phosphate buffer saline (PBS) were purchased from GIBCO (Grand Island, NY, U.S.A.). 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazidi-

um bromide (MTT) agent, Bovine serum albumin and gelatin were purchased from Sigma (St. Louis, MO, U.S.A.). Matrigel was purchased from Becton Dickinson (Bedford, MA, U.S.A.). DiffQuick was purchased from Dade Behring (Newark, DE, U.S.A.). Trizol was purchased from Life Technologies (Grand Island, NY, U.S.A.). Oligo poly dT was purchased from Roche Diagnostics (Indianapolis, IN, U.S.A.). Taq polymerase and deoxynucleotide triphosphate (dNTP) were bought from Takara. Murine moloney lentivirus (MMLV) reverse transcriptase was purchased from Promega (Madison, WI, U.S.A.). Urokinase type plasminogen activator (uPA), matrix metalloproteinase 2 (MMP2), tissue inhibitor of metalloproteinase 2 (TIMP2), glyceraldehydes-3-phosphate dehydrogenase (GAPDH) primers were purchased from GenoTech (Yusung, TJ).

**Animals** Male C57BL/6 mice, 4 weeks old, were purchased from Korea Research Institute of Chemical Technology. They were maintained under conventional conditions. Laboratory pellet chow (Samyang, Inc. Korea) and water were administered *ad libitum*.

**Aqueous Extract of Cambodian *Phellinus linteus*** Cambodian *Phellinus linteus* (CPL) was supplied from Life Trade Company. The mushroom was identified with the help of available literature and confirmed by Professor Nam-In Baek, Department of Botany, Kyunghee University. The specimen was deposited at Graduate School of East-West Medical Science, Kyunghee University, Korea (GSM. 021001). CPL (1 kg) was soaked into 2 fold volume of dis-

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tilled water in round flask and boiled for 2 h using extracting device. The extracted CPL was filtered with Whatman filter and concentrated with rotary vacuum evaporator (Eyela, Japan). This flask was placed in  $-84^{\circ}\text{C}$  deep freezer (Sanyo, Japan) for 24 h and then dried by freeze dryer (Eyela, Japan) for 12 h to obtain 17.75 g (yield=1.78%). It was dissolved with distilled water and filtered with  $0.2\ \mu\text{m}$  syringe filter. The solution was stored at  $4^{\circ}\text{C}$  until use.

**Cell Lines and Culture** B16BL6 melanoma cells (ATCC, CRL6322, U.S.A.) were purchased from ATCC. B16BL6 cells were cultured in Minimum essential medium containing 10% fetal bovine serum (FBS), 1% antibiotics (penicillin 100000 units/ml, streptomycin 10 mg/ml) and 2 g sodium bicarbonate at  $37^{\circ}\text{C}$  in  $\text{CO}_2$  incubator (Forma Scientific, Inc., U.S.A.) with 5%  $\text{CO}_2$  and 95% air.

**Analysis of ITS Nucleotide Sequences** DNA sequences of Cambodian mushroom were analyzed by Microbiotech Company (Pusan, Korea). For phylogenetic analyses, only the ITS1 and ITS2 sequences were used. After extracting DNA from Cambodian *Phellinus linteus*, specific part of internal transcribed spacer (ITS 1 and 2) was amplified and sequenced using auto sequencer. The sequence of amplified DNA product was used to search and align within National Center for Biotechnology (NCBI) database by BLAST.

**Cytotoxicity Assay** The cell viability was examined by MTT assay.<sup>12)</sup> B16BL6 cells were plated at  $1 \times 10^4$  cells/well in 96 well plate for 24 h. The cells were treated with various concentrations of CPL (125, 250, 500, 1000  $\mu\text{g/ml}$ ) for 24 h. MTT was added to each well and incubated for 4 h at  $37^{\circ}\text{C}$ . Formazan crystals were dissolved by addition of DMSO solution. The absorbance of each well was determined using the microplate reader (Molecular Devices Co., U.S.A.) at 570 nm.

**Tumor Cell Induced Platelet Aggregation Assay** Blood platelets were obtained from the Red Cross in Suwon. Platelet rich plasma (PRP) was obtained from platelet suspensions ( $2.5 \times 10^8/225\ \mu\text{l}$ ) following centrifugation at 850  $g$  for 15 min. PRP was preincubated for 2 min at  $37^{\circ}\text{C}$  in a cuvette of Chrono-log aggregometer (IL, U.S.A.). Platelet aggregation was initiated by the addition of B16BL6 cells ( $2 \times 10^5/225\ \mu\text{l}$ ) in control and the effect of CPL (125, 250, 500  $\mu\text{g/ml}$ ) on the platelet aggregation induced by B16BL6 cells was monitored.<sup>13)</sup> The inhibitory ratio of platelet aggregation was calculated as inhibitory percentage by CPL of untreated control at indicated concentrations by aggrolink software.

**Tumor Cell Adhesion Assay** Tumor cell adhesion assay was performed as described.<sup>14)</sup> Each well of 96 well plate was coated with 0.2% gelatin and incubated for 4 h. The plates were washed and incubated for 1 h with 1% bovine serum albumin to block unbounded surface. Prior to addition of the cells to each well, the B16BL6 cells ( $5 \times 10^4$ ) were preincubated with CPL (125, 250, 500  $\mu\text{g/ml}$ ) for 15 min at  $37^{\circ}\text{C}$ . The incubated cells were added to each well and incubated for 30 min at  $37^{\circ}\text{C}$  in 5%  $\text{CO}_2$ , 95% air. Unattached cells were removed by washing with PBS. The MTT assay was performed to calculate the number of attached cells. MTT was added to each well and incubated for 4 h at  $37^{\circ}\text{C}$ . Formazan crystals were dissolved by addition of DMSO solution. The absorbance of each well was determined using the microplate reader (Molecular Devices Co., U.S.A.) at

570 nm.

**Tumor Cell Invasion Assay** The Boyden chamber (Neuro Probe, Inc. U.S.A.) was used to evaluate the spontaneous invasion of B16BL6 cells as described.<sup>15)</sup> The method is based on the passage of cells across porous filters separating the upper and lower wells of the migration chamber. Polyvinyl-pyrrolidone-free polycarbonate filters ( $8\ \mu\text{m}$  pore size) were used in this experiment. The filters were coated with the reconstituted basement membrane Matrigel (50  $\mu\text{g}$ /filter). The cells ( $5 \times 10^5$ ) pre-cultured in FBS free-MEM medium in the absence or presence of CPL for 7 h were loaded onto the upper compartment of the Boyden chamber with various concentrations of CPL (125, 250, 500, 1000  $\mu\text{g/ml}$ ). FBS free MEM containing 0.1% bovine serum albumin was placed in the lower compartment of the Boyden chamber. The migration was allowed to occur in the absence (control condition) or in the presence of CPL in the medium of the upper and the lower compartment of the migration chamber. The chamber was incubated at  $37^{\circ}\text{C}$  for 7 h and the filters were removed and fixed in methanol. Non-migrated cells on the upper surface of the filter were removed with a cotton swab, while migrated cells, adherent on the lower filter surface, were stained with Diff-Quick (Mertz-Dade AG, Dade International, Milan, Italy) and counted under a light microscope ( $\times 400$  in 10 random fields) per each well. Each experiment was performed in triplicates. Migration values were expressed as means  $\pm$  S.D. of the number of migrated cells  $\times 100\%$ / total cells counted on the lower surface of filter.

**Experimental Lung Metastasis** Experimental lung metastasis of B16BL6 cells were assessed following intravenous inoculation of B16BL6 cells into syngeneic C57BL/6 mice. The B16BL6 cells were harvested from monolayer culture by trypsinization. The cell viability was determined and adjusted to  $7 \times 10^4$  in 0.2 ml PBS by trypan blue assay. The cells were injected into the lateral tail vein of mice 2 d after CPL treatment. CPL was orally administered at doses of 2, 20, and 50 mg/kg every other day for 14 d. The mice were killed 14 d after tumor inoculation. Their lungs were separated and fixed in Bouin's solution. Lung tumor colonies were counted under a dissecting microscope.

**RT-PCR Analysis** mRNA levels of uPA, MMP-2 and TIMP-2 were determined by an internal-based semiquantitative reverse transcriptase-polymerase chain reaction assay (RT-PCR), as previously described.<sup>16)</sup> B16BL6 cells were plated at  $5 \times 10^5$  cells/well in 6 well plates for 24 h. The cells were treated with various concentrations of CPL (250, 500, 1000  $\mu\text{g/ml}$ ) for 24 h. Total RNA was prepared from B16BL6 cells by using Trizol reagent. Total RNA (1.0  $\mu\text{g}$ ) was reverse transcribed by using oligo poly (dT) and MMLV reverse transcriptase. The mixture was incubated for 10 min at  $25^{\circ}\text{C}$ , then for 60 min at  $42^{\circ}\text{C}$  and finally for 5 min at  $99^{\circ}\text{C}$ . Specific primers were used to amplify cDNAs:

MMP-2 sense was 5'-TAGGCTTCTGCCCTGAGAC-3', while MMP-2 antisense was 5'-TCCAAACTTCACGCTCTTCA-3'(expected product 970 bp). TIMP-2 sense was 5'-TGCAGCTGCTCCCCGGTGCAC-3', whereas TIMP-2 antisense was 5'-TTATGGGTCCTCGATGTCGAG-3'(expected product 581 bp). uPA sense was 5'-CTGCCTGCCCTGGA ACTCTG-3', while uPA antisense was 5'-CCTTGCGTGTGGAGTTAAG-3'(expected product 477 bp).

GAPDH sense was 5'-GTGGATATTGTTGCCATCA-3',

while GAPDH antisense was 5'-ACTCATACAGCACCTC-AG-3' (expected product 700 bp). GAPDH was used in each experiment as an internal control. 30 cycles consisting of 30 s at 94 °C, 30 s at 59 °C and 30 s at 72 °C were used, and then followed by a 5 min incubation at 72 °C. Agarose gel electrophoresis was performed and the ethidium bromide-stained cDNAs were photographed under a U.V. transilluminator by using Polaroid positive/negative instant films. The amount of mRNA transcripts was analyzed by densitometry.

**Statistical Analysis** All values were expressed as means  $\pm$  S.D. Statistical significance was compared between each treated group and control by the Student's *t*-test. Results with  $p < 0.05$  were considered significantly different from control.

## RESULTS

**Analysis of ITS Nucleotide Sequences in Ribosomal DNA of Cambodian Mushroom** DNA sequences of Cambodian mushroom were analyzed. Cambodian mushroom turned out to be a species of *Phellinus linteus* with 99% homology of 621 DNA sequences of *Phellinus linteus* by the analysis of National Center for Biotechnology Information. Cambodian mushroom 1 to 621 is homologous with *Phellinus linteus* 690 to 70 except that thymine 657 of *Phellinus linteus* was changed to adenine 34 for Cambodian mushroom.

**Effect of CPL on the Cytotoxicity against B16BL6 Cells** To evaluate the cytotoxicity of CPL, MTT assay was performed. B16BL6 cells ( $1 \times 10^4$  cells/well) were plated in 96-well plate and treated with various concentrations of CPL for 24 h. The viability of B16BL6 cells was evaluated following MTT staining by ELISA. The cell viability was shown as  $104.2 \pm 1.7$ ,  $98.8 \pm 1.2$ ,  $96.5 \pm 2.0$  and  $89.5 \pm 0.7$  at various concentrations of CPL (125, 250, 500, 1000  $\mu\text{g/ml}$ ), respectively. Thus, CPL showed weak cytotoxicity on B16BL6 cells.

**Inhibitory Effect of CPL on Platelet Aggregation Induced by B16BL6 Cells** To examine the effect of CPL on platelet aggregation induced by B16BL6 cells, we employed TCIPA assay with a dual aggregometer. Platelet aggregation induced by B16BL6 cells was inhibited by various concentrations of CPL (125, 250, 500  $\mu\text{g/ml}$ ) as 40%, 95% and 96%, respectively, compared with untreated control (Fig. 1).

**Inhibitory Effect of CPL on the Adhesion of B16BL6 Cells** To evaluate the effect of CPL on the adhesion of tumor cells to gelatin, one of components of extracellular matrix, tumor cell adhesion assay was performed. The B16BL6 cells pretreated with CPL or PBS were incubated in 96-well plates coated with 0.2% gelatin and the number of unattached cells by CPL was compared with untreated control. CPL inhibited the adhesion of B16BL6 cells to 0.2% gelatin by 30%, 35% and 42% at doses of 125, 250 and 500  $\mu\text{g/ml}$ , respectively, compared with untreated control (Fig. 2).

**Inhibitory Effect of CPL on the Invasion of B16BL6 Cells** To examine the effect of CPL on the invasion of B16BL6 cells, we used *in vitro* tumor cell invasion assay using Boyden-chamber. CPL reduced the number of invaded B16BL6 cells into Matrigel up to  $71.7 \pm 5.7$ ,  $67.7 \pm 2.2$ ,  $31 \pm 14.1$ ,  $13.9 \pm 8.8$  at the concentrations of 125, 250, 500 and 1000  $\mu\text{g/ml}$ , respectively, compared with untreated con-

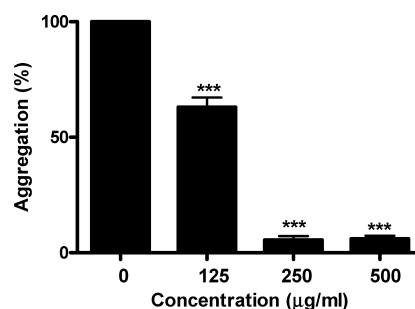


Fig. 1. Inhibitory Effect of CPL on Platelet Aggregation Induced by B16BL6 Cells

Normal human platelets ( $2.5 \times 10^8/225 \mu\text{l}$ ) were pre-incubated at 37 °C following a centrifugation at 1000 rpm for 30 min. B16BL6 cells ( $2 \times 10^5/225 \mu\text{l}$ ) were co-treated with various concentrations of CPL. Channel 1, control (PBS only); Channel 2, 125  $\mu\text{g/ml}$ ; Channel 3, 250  $\mu\text{g/ml}$ ; Channel 4, 500  $\mu\text{g/ml}$  of CPL. Each value represents mean  $\pm$  S.D. of three different experiments performed. \* Statistically significant value compared with control data (\*\*\*)  $p < 0.001$ .

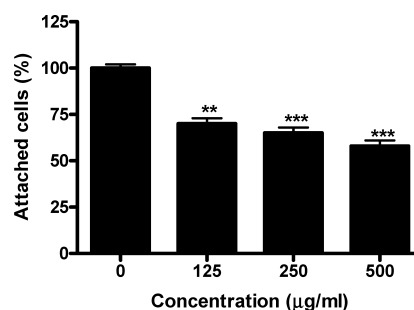


Fig. 2. Inhibitory Effects of CPL on the Adhesion of B16BL6 to Gelatin

96 well plate was coated with 0.2% gelatin and incubated for 4 h. The plates were washed and incubated for 1 h with 1% bovine serum albumin to block unbounded surface. The B16BL6 cells ( $5 \times 10^4$ ) were preincubated with CPL (125, 250, 500  $\mu\text{g/ml}$ ) for 15 min at 37 °C. The MTT assay was performed to calculate the number of attached cells. The absorbance of each well was determined using the microplate reader at 570 nm. \* Statistically significant value compared with control data. (\*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ )

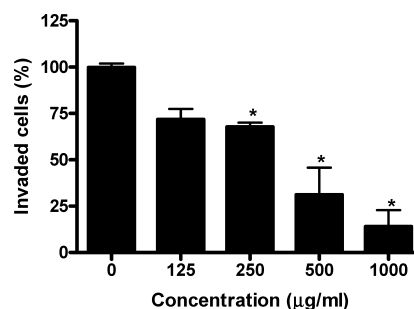


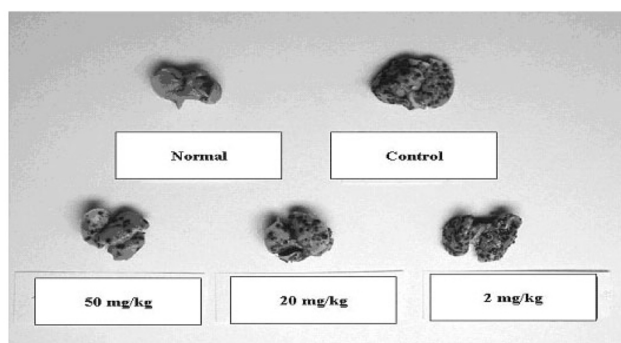
Fig. 3. Inhibitory Effects of CPL on the Invasion of B16BL6 Cells into Matrigel

The values were expressed as the percentage of cells adhered at the lower side of the migration filter in the presence of CPL compared with the number of untreated control cells (PBS alone) taken as 100%. B16BL6 cells ( $5 \times 10^5$ ), pretreated for 7 h with PBS alone or with CPL, were placed in the upper well of the migration chamber and incubated at 37 °C for 6 h. The filter was then removed and fixed in methanol. At the end of incubation, a number of invaded cells were counted under a light microscope ( $\times 400$  in 10 random fields) per each field. Each value represents mean  $\pm$  S.D. of 3 different experiments performed in triplicates. \* Statistically significant value compared with control data (\*  $p < 0.05$ ).

rol (Fig. 3).

**Inhibitory Effect of CPL on Pulmonary Metastasis by B16BL6 Cells** To investigate the inhibitory effect of CPL on pulmonary metastasis, the pulmonary colonization assay was employed. Oral administration of CPL dose-dependently

A



B

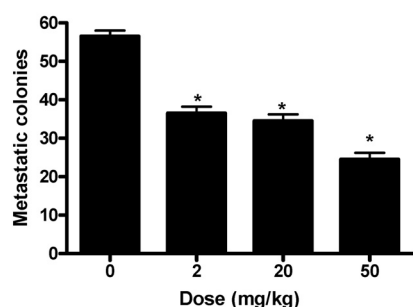


Fig. 4. Inhibitory Effects of CPL on Pulmonary Metastasis in C57/BL6 Mice i.v Injected by B16BL6 Cells

Seven mice per group were intravenously injected by B16BL6 cells ( $7 \times 10^4$ ). CPL was orally administered at doses of 2, 20, and 50 mg/kg every other day for 14 d. The mice were killed on day 14 following tumor inoculation. Their lungs were separated and fixed in Bouin's solution. Lung tumor colonies were counted under a dissecting microscope. Photographs of lung metastatic colonies in control and CPL treated groups (A). The tumor metastatic colonies in the lungs were counted (B). Each value represents mean  $\pm$  S.D. of two experiments performed. \* Statistically significant value compared with control data (\* $p < 0.05$ ).

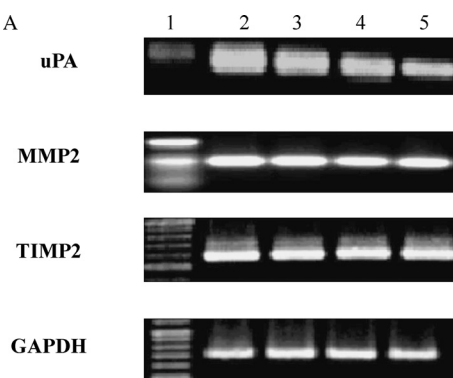
suppressed the number of metastatic colonies in lungs of C57/BL6 mice following intravenous inoculation of B16BL/6 cells by 36%, 36.9% and 55.5% at doses of 2, 20 and 50 mg/kg, respectively, compared with untreated control (Fig. 4).

**Effect of CPL on the Expressions of uPA, MMP-2 and TIMP-2** To evaluate the effect of CPL on the expression of uPA, MMP-2 and TIMP-2 in B16BL6 cells, RT-PCR was performed. mRNA level of uPA was effectively down-regulated, while the expressions of MMP-2 and TIMP-2 were not significantly affected by CPL compared with control (Fig. 5).

## DISCUSSION

Cancer metastasis is a highly coordinated multistep process in which cancer cells undergo extensive interactions with various host cells before they establish a secondary metastatic colony. Many morphological studies have documented the close association of circulating tumor cells with host platelets.<sup>17)</sup> There are several evidences that tumor cell-platelet interactions (*i.e.*, TCIPA) significantly contribute to hematogenous metastasis.<sup>18,19)</sup> Clinically, cancer patients are characterized by a variety of thromboembolic disorders including thrombocytosis. Pharmacologically, various anti-

A



B

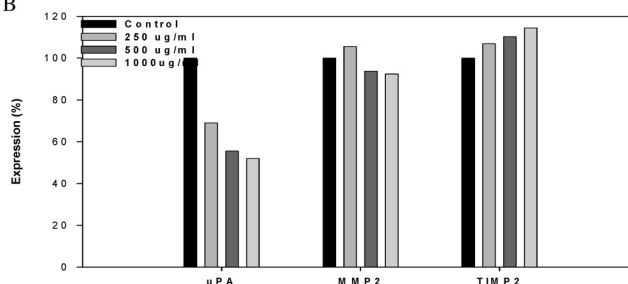


Fig. 5. Effect of CPL on the Expressions of uPA, MMP-2 and TIMP-2 mRNA in B16BL6 Cells

Total RNA (1.0  $\mu$ g) was reverse transcribed by using oligo poly (dT) and MMLV reverse transcriptase. The mixture was incubated for 10 min at 25  $^{\circ}$ C, then for 60 min at 42  $^{\circ}$ C and finally for 5 min at 99  $^{\circ}$ C. Primers of uPA, MMP-2 and TIMP-2 were used to amplify cDNAs. After 25 cycles of PCR amplification, 10  $\mu$ l aliquots were taken and separated on a 1% (w/v) agarose gel containing ethidium bromide (1  $\mu$ g/ml). The PCR products were quantified densitometrically by a laser scanner. Markers: lane 1: 100 bp marker, lane 2: Control, lane 3: 250  $\mu$ g/ml, lane 4: 500  $\mu$ g/ml, lane 5: 1000  $\mu$ g/ml of CPL (A). Results of laser densitometry were shown in the bar graph (B).

platelet agents/anti-coagulants have demonstrated potent inhibitory effects on tumor cell-platelet interactions as well as spontaneous or experimental metastasis.<sup>17)</sup> Experimentally, interference with many of the intermediate steps of tumor cell-platelet interactions has resulted in the inhibition of platelet aggregation induced by tumor cells and subsequently blocked cancer metastasis.<sup>20,21)</sup>

Cambodian mushroom has been used for the treatment of cancer as a folk medicine. However, little was known on the botanical and scientific evidences about Cambodian mushroom. By DNA sequence analysis of Cambodian mushroom, Cambodian mushroom turned out to have 99% homology of 621 DNA sequences of *Phellinus linteus* by comparison with National Center for Biotechnology Information. Therefore, Cambodian mushroom was identified as a species of *Phellinus linteus*.

CPL was expected to have anti-tumor activity, considering that *Phellinus linteus* is a well-known species of the genus *Phellinus*, which attracts great attention due to its anti-tumor effect and other medicinal values.<sup>5)</sup> Thus, in the present study, we tried to investigate the anti-metastatic activity of CPL associated with tumor thrombosis by tumor cell induced platelet aggregation, tumor cell adhesion, invasion and protease degradation.

CPL did not show significant cytotoxicity on B16BL6 cells suggesting CPL may have another biological action to exert anti-tumor activity.

Platelet interaction with tumor cells is a sequential process

which involves two general types of mediators, *i.e.*, membrane-bound molecules (adhesion molecules) and soluble release products to promote metastasis.<sup>20,22</sup> Mechanistically, platelets may contribute to metastasis by stabilizing tumor cell arrest in the vasculature, stimulating tumor cell proliferation, promoting tumor cell extravasation by potentiating tumor cell-induced endothelial cell retraction, and enhancing tumor cell interaction with the extracellular matrix (ECM).<sup>17</sup>

In our study, CPL inhibited platelet aggregation induced by B16BL6 cells in a concentration dependent manner. These results indicate that CPL may disrupt the interaction between tumor cell and platelets, *i.e.*, the function of tumor thrombosis or adhesion molecules.

Adhesive interaction of tumor cells to ECM and its invasive action into ECM are known to be fundamental events in tumor metastatic process.<sup>23</sup> Here, CPL inhibited the adhesion of B16BL6 cells to 0.2% gelatin, one of the ECM components as well as effectively suppressed the invasion of B16BL6 cells in a concentration dependent manner.

ECM consists of interstitial components such as collagen, laminin, vitronectin and fibronectin. Cell surface-binding to extracellular interstitial collagen may trigger intracellular signaling and alteration in morphology and gene expression. The reduction in interstitial collagenase is closely associated with the loss of the invasive phenotype.<sup>24</sup> Thus, it is necessary to study further the effect of CPL or active components on the interstitial components of ECM or integrin, ECM receptor.

To confirm *in vitro* anti-invasive activity of CPL, experimental pulmonary metastasis assay was applied. Consistent to *in vitro* data, CPL dose-dependently inhibited the pulmonary metastasis of B16BL6 up to 55.5% of untreated control at a dose of 50 mg/kg.

The serine protease urokinase plasminogen activator (uPA) converts inactive plasminogen into plasmin and therefore plays a key role to initiate a cascade of proteolytic steps accumulating in the degradation of the extracellular matrix (ECM). uPA is found in cellular structures at the leading edge of migrating cells that are involved in adhesion, migration, invasion, intravasation and metastasis.<sup>25</sup> Interestingly, CPL down-regulated the expression of uPA in a concentration dependent manner.

MMP-2 is a proteolytic enzyme capable of degrading the structural support network for normal and malignant cells, promoting neoplastic cell invasion and metastasis, while TIMP-2 maintains connective tissue integrity by modulating MMP activity.<sup>26</sup> However, unexpectedly, CPL did not exhibit the statistical significance, though CPL tended to inhibit the expression of MMP-2 and activate TIMP-2 by RT-PCR analysis.

Considering that *in vitro* and *in vivo* data sometimes may be different by crude drugs consisting of complex components, we have to continue to study the biological activity or molecular mechanism with active components isolated from CPL to get reproducibility.

In summary, aqueous extract of Cambodian *Phellinus linteus* may inhibit experimental metastasis of B16BL6 cells in mice at least partly *via* regulation of uPA system associated with tumor cell induced platelet aggregation. However, it is still necessary to isolate active ingredients from Cambodian *Phellinus linteus* and compare the biological activity with another species of *Phellinus linteus* from different origin or harvest time.

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