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ScienceDirect
Food and Chemical Toxicology 44 (2006) 1890–1896
www.elsevier.com/locate/foodchemtox

Inhibition of tumor invasion and metastasis by aqueous extract of the radix of *Platycodon grandiflorum*

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Received 27 December 2005; accepted 8 June 2006

Abstract

Platycodon grandiflorum is a traditional oriental herbal medicine that is known for its immunostimulatory and anti-tumor effects. This study examined the anti-metastatic activities of an aqueous extract from the root of *P. grandiflorum* (Changkil: CK) using *in vitro* and *in vivo* metastasis assays. CK inhibited the invasion of B16-F10 melanoma cells through a reconstituted basement membrane (Matrigel)-coated filter, and strongly inhibited the adhesion of B16-F10 melanoma cell to extracellular matrices such as Matrigel, fibronectin and laminin substrates. CK also inhibited an experimentally induced lung cancer and prolonged the survival time *in vivo*. In addition, CK augmented NK cell activity. These results show that CK can reduce the extent of a lung metastasis of B16-F10 melanoma cells by inhibiting the adhesion of tumor cells to the basement membrane possibly and activating NK cells.

Keywords: *Platycodon grandiflorum*; Adhesion; Invasion; Metastasis; NK cell

1. Introduction

A complex series of steps is needed for the successful establishment of a tumor metastasis (Onn and Herbst, 2003; Kawaguchi, 2005). The tumor invasion of a basement membrane is also an important step, which involves the adhesion of tumor cells to the extracellular matrix (ECM) components followed by the degradation of the ECM (Cavallaro and Christofori, 2001). Several attempts have been made to inhibit tumor metastasis preventing the formation of tumors and tumor invasion using herbs (Ha et al., 2004; Yang et al., 2003). Many herbs have been examined in the biomedical area on account of their immunomodulating activity. The enhancement of the host immune responses has been recognized as a possible means of inhibiting tumor growth without harming the host. Therefore, many studies have been undertaken to discover immunostimulatory materials from a variety of sources. It has been reported that immunostimulatory materials isolated from various natural sources have anti-tumor activity by stimulating the immune system (Han et al., 1998; Loeffler et al., 2005; Zhang et al., 2005).

Herbs have recently become attractive as physiologically functional foods, as well as a source material for the development of drugs. Herbal medicines derived from plant extracts are increasingly being used to treat a wide variety of clinical conditions, with relatively little knowledge of their modes of action. Platycodi radix, which is the root of *Platycodon grandiflorum* A. DC (Campanulaceae) (four years old), has been used both as a food and in traditional oriental medicine to treat adult diseases, such as bronchitis, asthma, pulmonary tuberculosis, hyperlipidemia, and

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doi:10.1016/j.fct.2006.06.009

inflammatory diseases, as well as a sedative. Its biological significance has previously been reviewed (Lee, 1973). It was previously reported that Changkil (CK), which is an aqueous extract from the root of *P. grandiflorum* cultivated for more than 20 years (Lee, 1991), prevented hypercholesterolemia and hyperlipidemia (Kim et al., 1995) and enhanced some of the functions of macrophages, such as proliferation, spreading ability, phagocytosis, cytostatic activity, NO secretion, as well as the gene expression of TNFα, IL-1β, and IL-6 (Choi et al., 2001a,b). Recently, it was reported that CK had antioxidant effects, hepatoprotective effects and prevented the progress of hepatic fibrosis in rats (Lee et al., 2001; Lee and Jeong, 2002; Lee et al., 2004a,b). In addition, it was also reported that CK activated macrophages via Toll-like receptor 4 (Yoon et al., 2003).

Although CK is believed to augment the immune response by modulating the macrophage function, the precise mechanism for the augmentation of cell-mediated immunity is unclear. Furthermore, other biological properties of CK, such as an effect on tumor invasion and metastasis, are still unknown. This study investigated the anti-tumor activity of CK with respect to the therapeutic inhibition of tumor metastasis in experimental metastasis models including the invasion of Matrigel *in vitro* and on a lung metastasis produced by B16-F10 melanoma cells in syngeneic mice. In addition, this study analyzed the mechanism for its anti-metastatic effect in view of enhancement of the host defense system against tumors through the activation of natural killer (NK) cells. The results show that CK has significant anti-metastatic activity in mice.

2. Materials and methods

2.1. Reagents

The RPMI 1640 media and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA, USA). The Calcein-AM was acquired from Molecular Probes (Eugene, OR, USA). Matrigel was obtained from Collaborative Research Inc. (Bedford, MA, USA). WST-1 Cell Counting Kit was purchased from Wako Pure Chemical Industries (Osaka, Japan). Unless otherwise stated, all other reagents were obtained from Sigma (St. Louis, MO, USA).

2.2. Preparation of CK

Aqueous extract (CK) from the root of *P. grandiflorum* (22 years old), supplied by Jangsaeng Doraji Co., Ltd., Chinju, South Korea, was prepared as described previously (Lee et al., 2001; Lee and Jeong, 2002): powdered root was added to distilled water (5 ml/g) and the mixture maintained at 90 °C for 10 h, cooled to room temperature, then filtered, and lyophilized. The yield of lyophilized residue corresponded to 33.5% (33.5 g of residue for each 100 g of original dry roots). The pale-yellow extract was dissolved directly in sterilized saline. The composition of CK was shown previously (Kim et al., 1995).

2.3. Animals

Five to six week old male C57BL/6 mice were purchased from the Dae Han Laboratory Animal Research and Co. (Daejeon, Korea). The animals were provided with Purina Rodent Chow and tap water ad libitum, and were maintained in a controlled environment at 21 ± 2 °C and 50 ± 5% relatively humidity with a l2 h dark/light cycle. The mice were acclimatized for at least 1 week prior to use. All the animal experiments were performed according to the rules and regulations of the Animal Ethics Committee, Chosun University.

2.4. Cell cultures

The highly invasive and metastatic murine melanoma cell line, B16-F10, and the murine lymphoma cell line, YAC-1 (natural-killer-cell-sensitive target cells), were maintained in RPMI 1640 containing 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified 5% CO_2 atmosphere.

2.5. In vitro invasion assay

The invasive activity of the tumor cells was examined in a Transwell cell culture chamber using a method described elsewhere (Albini et al., 1987). Polyvinylpyrrolidone-free polycarbonate (PVPF) filters of 8.0 μm pore size were coated with 500 μg/ml of Matrigel and placed in Transwell well chambers. The coated filters were washed thoroughly in PBS and dried immediately before use. Ten percent FBS-RPMI 1640 was placed in the lower chamber, and B16-F10 cells (2×10^5/chamber) in RPMI 1640 were placed in the upper chamber. The CK solution was added to the upper chamber and incubated for 4.0 h at 37 °C in 5% CO_2. The number of the invaded cells through Matrigel-coated PVPF filter was measured by counting cells stained with 0.2% crystal violet solution.

2.6. Microassay for cell adhesion

The cell attachment assay was carried out in 96-well plates using a slight modification of a method described elsewhere (Saiki et al., 1989). The wells were precoated with 50 μl of 5 μg/ml fibronectin, 50 μl of 10 μg/ml Matrigel, or 50 μl of 40 μg/ml laminin overnight at room temperature and blocked with 0.2 ml of RPMI 1640/well containing 3% BSA for 1 h at 37 °C. The cells were resuspended in RPMI 1640 containing 0.1% BSA, added (5×10^5/ml, 0.2 ml/well) to each well and the CK was added. This suspension was incubated at 37 °C for 1 h. The wells were washed twice with warm PBS to remove the unattached cells, and the attached cells were then stained with a 0.2% crystal violet aqueous solution in 20% methanol for 10 min. Once stained, the cells were dissolved in 200 μl of a 1% sodium dodecyl sulfate (SDS) solution, and the optical density was measured at 560 nm using a microplate reader (Varioskan, Thermo Electron Co., Vantaa, Finland).

2.7. Cytotoxicity assay

The level of cell growth was examined using a WST-1 Cell Counting Kit. Briefly, the B16-F10 cells (5×10^3/well) in 10% FBS-RPMI 1640 were seeded into the 96-well plates. After incubation for 24 h, various concentrations of CK were added to the well, and the plates were incubated at 37 °C for an additional 24 h. Doxorubicin hydrochloride was used as the inhibitory control. A WST-1 solution (10 μl) was added to each well and incubated at 37 °C for 4 h before ending the experiment. The absorbance at 450 nm was measured using a microplate reader.

2.8. In vivo experimental lung metastasis assay

The log-phase cell cultures of the B16-F10 cells were harvested, washed with serum-free RPMI 1640 and resuspended to give the appropriate concentrations in PBS. An amount of 0.2 ml of the resultant B16-F10 cell suspension (5×10^5 cells) was injected via the tail vein of the C57BL/6 mice (day 0). The CK was suspended in sterilized saline and administered orally to the mice simultaneously with the induction of metastasis. The treatment was continued daily for 7 days. The animals were randomly divided into three groups consisting of 12 mice each. Groups 1–3 were injected with B16-F10 cells. Group 1 was injected with B16-F10 cells alone receiving only the vehicle. Groups 2 and 3 were administrated 20 and 100 mg/kg CK,

respectively. The body weights were monitored each day. Six mice from each group were sacrificed 14 days after being inoculated with the tumor cells, and the lungs were fixed in Bouin's solution (saturated picric acid:20% formalin neutral buffer solution:acetic acid = 15:5:1). The number of B16-F10 colonies present on the surface of each set of lungs was determined by a visual inspection using a stereoscopic dissecting microscope. The remaining six mice in all the groups were observed to determine their survival. The survival was monitored for more than 2 months.

2.9. Isolation of splenic lymphocytes

CK was suspended in sterilized saline and administered orally to the mice daily for 3 days. The splenic lymphocytes were then isolated using a method described elsewhere (Kimura and Okuda, 2001). The cell viability was always greater than 90%, as judged by Trypan blue dye exclusion.

2.10. Natural killer (NK) cell activity assay

The NK cell activity was determined by performing a calcein-AM release assay according to a slight modification of a method reported elsewhere (Ayalon et al., 1998). The YAC-1 cells (2×10^5 cell/ml) were labeled with 10 μg/ml calcein-AM in serum-free RPMI 1640 for 30 min. After washing three times with complete RPMI 1640 medium, 0.1 ml of the labeled YAC-1 cells were added to each well of a 96-well plate. The effector cells, splenic lymphocytes (0.1 ml), were added to each well to produce various effector-to-target (E/T) ratios. This plate was incubated for 4 h at 37 °C. After centrifugation, 0.1 ml of the cell-free supernatant was removed, and the fluorescence intensity of the supernatant was measured using a spectrofluorometer (Varioskan, Thermo Electron Co., Vantaa, Finland) with excitation at 500 nm and emission at 540 nm. The specific cytolytic activity (as percent lysis) was calculated as follows: % of the specific lysis $= (E - S)/(T - S) \times 100$ (where E is the fluorescence released in the experimental cultures of target cells and effector cells; S is the spontaneous fluorescence released in the cultures containing only the target cells; T is the total fluorescence).

2.11. Statistical analysis

Results are reported as means ± SD ANOVA was used to evaluate the difference between multiple groups. If a significant difference was observed between groups, Dunnet's 't' test was used to compare the means of two specific groups. Survival data were analyzed by Fisher's exact test and Kaplan–Meier survival test. A P value of <0.01 was considered to be statistically significant.

3. Results

3.1. Effect of CK on tumor cell invasion

An *in vitro* invasion assay was performed using the highly invasive and metastatic murine melanoma cell lines, B16-F10, to determine if CK can inhibit a tumor metastasis. Fig. 1 shows the inhibitory effect of CK on the invasion of B16-F10 cells into a reconstituted basement membrane (Matrigel) in Transwell cell culture chambers. The invasion of B16-F10 cells into the Matrigel/fibronectin-coated filters was inhibited by CK in a concentration-dependent manner, and a 50% reduction was achieved at about 20 μg/ml.

3.2. Effect of CK on tumor cell adhesion to ECM

The effect of CK on the adhesion of B16-F10 cells to ECM components was examined because tumor cell adhesion to the ECM is believed to be a fundamental step in tumor invasion (Fig. 2). The B16-F10 cells were treated with CK at concentrations ranging from 1 to 100 μg/ml. CK inhibited the adhesion of B16-F10 melanoma cells to the Matrigel ($IC_{50} = 43$ μg/ml) and fibronectin ($IC_{50} = 62$ μg/ml) in a concentration-dependent manner. In particular, CK strongly inhibited the adhesion of B16-F10 cells to laminin, and the half maximal inhibition of cell adhesion

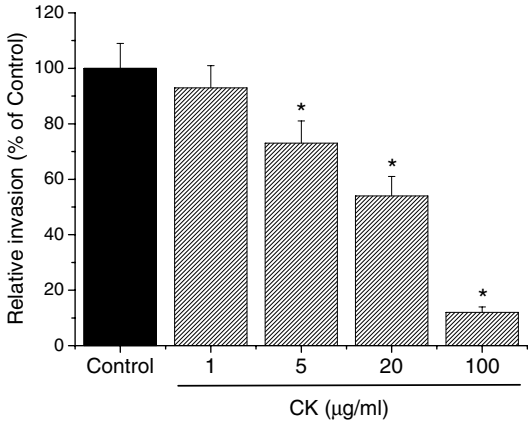

Fig. 1. Effect of CK on a B16-F10 melanoma cell invasion of Matrigel. B16-F10 melanoma cells were seeded onto the filters precoated with Matrigel on the upper surface and fibronectin was coated on the lower surface in the Transwell chambers in the presence or absence of CK. The cells that had invaded to the lower surface were stained with a crystal violet solution and measured by counting the number of stained cells after 4 h incubation. Invasion is expressed as % of the untreated control. Each bar shows the mean ± SD of three independent experiments that were performed in triplicate. $^*P < 0.01$ compared with the control.

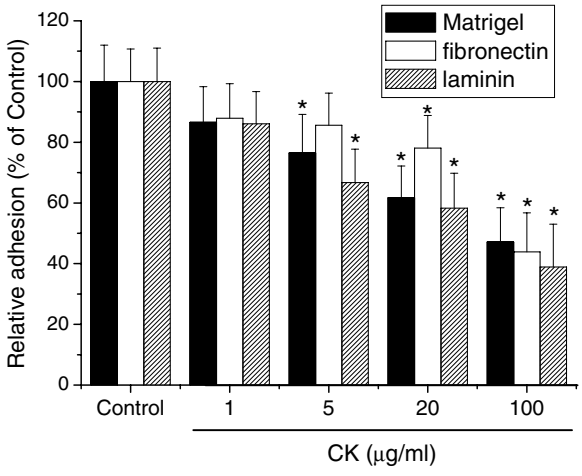

Fig. 2. Effect of CK on the adhesion of B16-F10 melanoma cells to the ECM components. B16-F10 melanoma cells were added to the microculture wells precoated with Matrigel, fibronectin, or laminin in the presence or absence of CK. After 1 h incubation, the non-adherent cells were washed away and the adherent cells were stained with crystal violet. After extensive washing, the stained cells were lysed with sodium dodecyl sulfate and the absorbance was measured at 560 nm. Tumor cell adhesion is expressed as % of the untreated control. Each bar shows the mean ± SD of three independent experiments that were performed in triplicate. $^*P < 0.01$ compared with the control.

was observed at 31 μg/ml. In the next series of experiments, the inhibitory mechanism of CK on the adhesion of B16-F10 cells to the laminin substrate was further examined. B16-F10 cells were incubated with CK at concentrations ranging from 1 to 100 μg/ml for 1 h on ice, and then washed with PBS. In other experiments, laminin-coated substrates were incubated with CK for 3 h at 37 °C, and washed with PBS. Fig. 3 shows that the pretreatment of B16-F10 cells with CK prevented cell attachment to the laminin substrate in a concentration-dependent manner. However, pretreatment of the substrates with CK had no inhibitory effect.

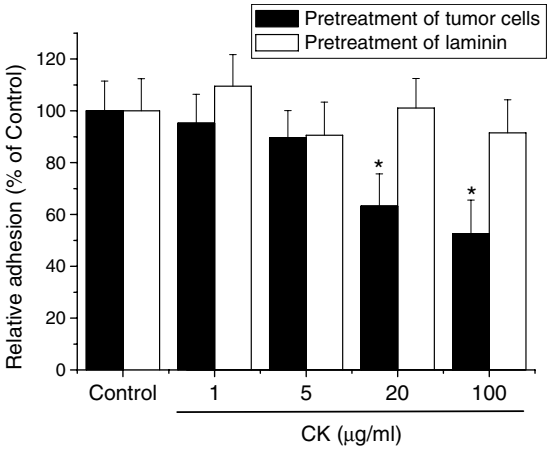

Fig. 3. Inhibition of tumor cell adhesion to laminin by pretreating the B16-F10 cells with CK. B16-F10 melanoma cells were pretreated with CK for 1 h and washed with PBS. Pretreatment of tumor cells: the cells were added to microculture wells precoated with laminin. Pretreatment of laminin; the microculture wells precoated with laminin were treated with CK for 1 h and then washed with PBS. After 1 h incubation, the non-adherent cells were washed away and the adherent cells were stained with crystal violet. After extensive washing, the stained cells were lysed by sodium dodecyl sulfate and the absorbance was measured at 560 nm. Tumor cell adhesion is expressed as % of the untreated control. Each bar shows the mean ± SD of three independent experiments performed in triplicate. $^{*}P < 0.01$ compared with the control.

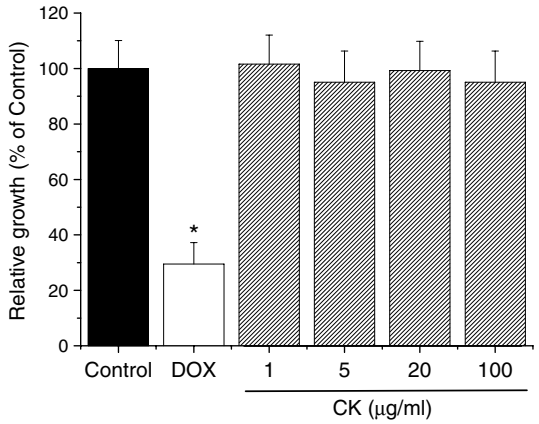

Fig. 4. Effect of CK on the growth of B16-F10 melanoma cells. The B16-F10 cells were incubated with various CK concentrations or doxorubicin hydrochloride (DOX; 172 μM) for 24 h. A WST-1 solution was added to each well and incubated for 4 h before the termination. The absorbance at 450 nm was measured using an immunoreader. Tumor cell growth is expressed as % of the untreated control. Each bar shows the mean ± SD of two independent experiments that were performed in triplicate. $^{*}P < 0.01$ compared with the control.

3.3. Cytotoxic effect of CK on the B16-F10 melanoma cells

A WST assay was performed to determine if the anti-adhesion effect of CK was caused by a false positive due to cytotoxicity. Fig. 4 shows that CK had no inhibitory effect on the growth of B16-F10 cells after 24 h incubation at the concentrations used in this study (1–100 μg/ml). On the other hand, doxorubicin hydrochloride, which was used as a positive control, potently inhibited cell growth *in vitro*. CK showed slight cytotoxicity at concentrations exceeding 800 μg/ml (data not shown). These results suggest that the inhibition of tumor cell adhesion and invasion is not due to the cytotoxicity of CK.

3.4. Effect of CK on experimental lung metastasis and survival

Since the above results demonstrated that CK caused a marked reduction of tumor invasion *in vitro*, this study examined the effect of CK on *in vivo* experimental lung metastasis produced by an intravenous injection of B16-F10 melanoma cells using C57BL/6 mice. Table 1 shows that there was a concentration-dependent reduction in the number of lung metastases in the simultaneous CK-treated mice compared with the untreated controls. In particular, a treatment with 100 mg/kg CK resulted in approximately 65% inhibition of the lung metastases (Table 1). At this dose level, there was no effect on the body weight nor were there any other clinical signs of toxicity (data not shown). These results demonstrate that CK orally administered has dose-dependent anti-metastatic activity. The survival time of the mice treated with 100 mg/kg was more than double that of the control group (Table 1).

Table 1
Effect of CK on lung colonization of B16-F10 melanoma cells and survival of the mice[a]

Treatment	Number of colony	Percent inhibition	Number of days survived	Percent ILS[b]
Control	275 ± 35	–	29.5 ± 4.3	–
CK 20 mg/kg	167 ± 24*	39.3	38.4 ± 5.7*	130.1
CK 100 mg/kg	94 ± 13*	65.8	62.6 ± 8.2*	212.2

Values are mean ± SD.

* $P < 0.01$ compared with the control.

[a] The lungs were dissected and observed for any metastases on the 14th day after inducing a B16-F10 melanoma. CK was started simultaneously with the tumor cell inoculation through the lateral tail vein (7 times at 24 h interval, p.o).

[b] Increase in life span $= (T/C) \times 100$, where T and C are the mean number of days survived by the treated and control (vehicle-treated) group of animals, respectively.

3.5. Effect of CK on NK cell activity in vivo

The effect of CK treatment on the innate host defense mechanisms, particularly NK cell augmentation, was examined because the simultaneous administration of CK was effective in preventing a tumor metastasis. The augmentation of NK cells has also been shown to suppress tumor metastasis (Pross and Lotzova, 1993; Whiteside and Herberman, 1995). In this study, CK was administered daily for 3 days, and the NK activity was examined 1 day after the final dose using splenic lymphocytes as the effector cells with YAC-1 as the target cells. The results demonstrated that CK significantly enhanced the splenic NK cell activity in a dose-dependent manner (Fig. 5A). The splenic NK cell activity was also increased simultaneously, resulting in a peak activity 3 days after the final injection (Fig. 5B). Therefore, CK activates NK cells *in vivo*.

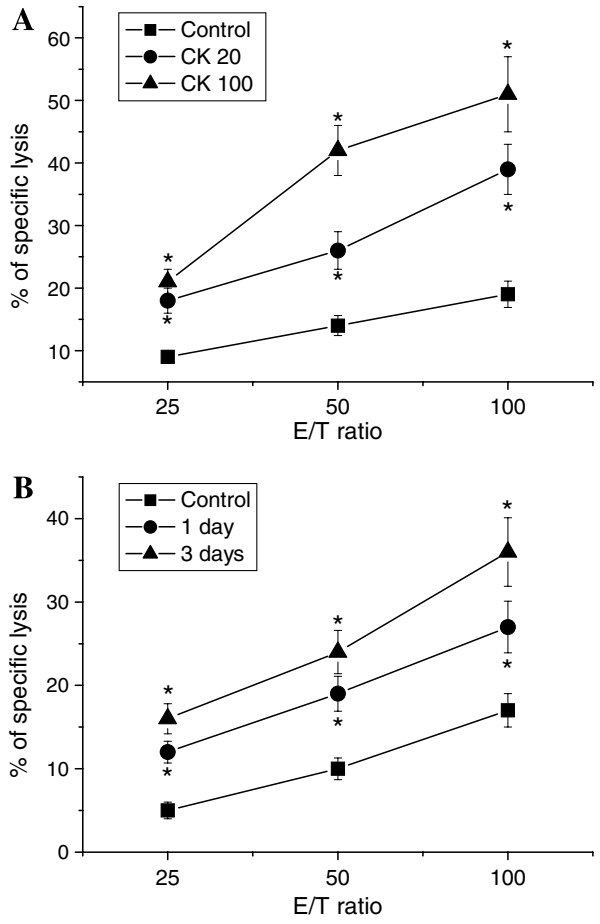

Fig. 5. CK activation of NK cells *in vivo*. (A) C57BL/6 mice were administered orally with saline (control), CK 20, or CK 100 mg/kg daily for 3 days. One day after the final dose of CK, the splenic lymphocytes were used in the cytolytic assays against the YAC-1 target cells at various effector/target (*E*/*T*) ratios as indicated. The results are expressed as the mean percentage specific lysis ± SD of three independent experiments that were performed in triplicate. (B) The cytolytic activity was assessed against YAC-1 target cells. CK at 20 mg/kg was administered orally daily for 3 days, and splenic lymphocytes were then prepared 1 day or 3 days after the final dose. The results are expressed as the mean percentage specific lysis ± SD from three independent experiments that were performed in triplicate. $^{*}P < 0.01$ compared with the control.

4. Discussion

Several anti-metastatic drugs or compounds for cancer have been found in natural products. Paclitaxel was identified as a component of the Pacific yew, *Taxus brevifolia*, and is used as an anticancer drug (Colomer, 2004). Curcumin and catechin were reported to strongly inhibit lung metastases induced by melanoma cells in mice (Menon et al., 1999). All these compounds are derived from plants. This study showed that CK significantly inhibited an experimental lung metastasis produced by an injection of B16-F10 melanoma cells in a dose-dependent manner (Table 1).

Tumor cell invasion to the ECM is an important step in the process of tumor metastasis, and involves the attachment of tumor cells to ECM (Liotta et al., 1991; Cavallaro and Christofori, 2001). The ECM proteins promote cell adherence by binding to the cell surface receptor, integrin (Kleinman et al., 1986; Kramer et al., 1989). This study demonstrated that CK inhibits the invasion of B16-F10 cells using a reconstituted basement membrane, Matrigel, *in vitro* (Fig. 1). An *in vitro* attachment assay showed that CK successfully blocked B16-F10 melanoma cells adhesion to the ECM proteins, such as Matrigel, fibronectin, and laminin, in a concentration-dependent manner (Fig. 2). The fact that short-term pretreatment of B16-F10 cells with CK blocked the adhesion to laminin (Fig. 3) suggests that the inhibition of cell adhesion to laminin substrate is associated with the binding of CK to the tumor cell surface rather than to the laminin substrate. However, the detailed mechanism is still unclear. One possible explanation is that CK binds to the laminin receptors such as the integrins on the tumor cell surface, which leads to the inhibition of tumor cell adhesion and invasion. CK did not show any cytotoxicity at the concentrations used in this study for the 24 h incubation (Fig. 4). This suggests that the anti-adhesion and anti-invasion effects of CK were not due to cytotoxicity.

There are many reports demonstrating the anti-tumor activity of biologically active compounds isolated from various sources via the stimulation of the immune system. Angelan isolated from *Angelica gigas* Nakai (Han et al., 1998) and *Tinospora cordifolia* (Leyon and Kuttan, 2004) potentiated the *in vivo* immune functions and increased the survival times of the mice implanted with B16-F10 melanoma cells and inhibited an experimental metastasis of this cells. Song et al. (2002) reported the immunostimulatory and anti-tumor effect of ginsan isolated from *Panax ginseng*. These reports suggest that the immunostimulatory active compounds isolated from various natural sources are good candidates for the development of anti-tumor agents.

Many experimental and clinical studies have shown that natural immunity plays an important role in both immunosurveillance and blockade of a metastasis from primary tumors (Schantz et al., 1987; Pollack and Hallenbeck,

1982). Long and short-term *in vitro* experiments have indicated that CK is neither directly toxic to tumor cells nor inhibits cell proliferation (Fig. 4). These results are indicative of the involvement of the immune system in the reduction of the metastatic potential of the CK-treated animals. Furthermore, the tumor reduction obtained may be mediated by B-cells or non-specific immune cells such as NK cells because CK selectively activates B cells and macrophages but not *T* cells (Han et al., 2001). These results suggest that CK might modulate the innate host defense mechanisms. In addition, the relevant effectors responsible for the natural immunity against tumors have been identified to be NK cells (Barlozzari et al., 1985; Pross and Lotzova, 1993; Whiteside and Herberman, 1995). Indeed, many investigators have reported that the activation of NK cells by immunostimulants leads to a reduction in the metastatic colonization of tumors (Herberman, 1984; Hanna, 1985; Leyon and Kuttan, 2004; Kamiryo et al., 2005). In fact, the tumor incidence and metastasis are higher in mice that are deficient in NK cells (Kim et al., 2000). In addition, *o*-galactosylceramide was reported to increase the number of NK cells *in vivo* and inhibit tumor metastasis (Nakagawa et al., 1998). Therefore, a more potent NK-augmenting agent has potential anti-tumor activity, but the appropriate molecular target has not yet been identified. Therefore, this study analyzed the mechanism of the inhibitory effects of CK on tumor metastasis in terms of NK cell activation. CK significantly augmented the NK activity *in vivo* (Fig. 5).

In conclusion, CK showed anti-metastatic effects both *in vitro* and *in vivo*. These results suggest that the anti-metastatic activity of CK may be due to blocking adherence and the augmentation of the NK cell activities against cancer cells. However, further research is needed to determine if the anti-metastatic activity of CK is due to blocking integrin-mediated adherence *in vivo*. CK might provide a promising basis for improved therapeutic approaches to tumor invasion and metastasis. It may be particularly well suited as a supportive agent in anticancer therapy after the surgical removal of a tumor mass and the recurrence of cancer.

Acknowledgements

The authors are grateful to Jangsaeng Doraji Co., Ltd., Chinju, South Korea for providing the root of *P. grandiflorum*. This study was supported by grants of the Korea Health 21 R&D Project, Ministry of Health & Welfare, Republic of Korea (02-PJ9-PG3-21600-0005), Research Center for Proteineous Materials by KOSEF, and the Regional Technology Innovation Program of the Ministry of Commerce (No. RTI 04-03-07), Industry and Energy (MOCIE).

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