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

Kyung Jin Lee [a,1], Ji Young Kim [a,1], Jae Ho Choi [a], Hyung Gyun Kim [a], Young Chul Chung [b], Seong Hwan Roh [c], Hye Gwang Jeong [a,*]

[a] *Department of Pharmacy, College of Pharmacy, Research Center for Proteineous Materials, Chosun University, 375 Seosuk-dong, Kwangju 501-759, South Korea*
[b] *Division of Food Science, Chinju International University, Chinju, South Korea*
[c] *Jangsaeng Doraji Research Institute of Biotechnology, Jangsaeng Doraji Co., Ltd., Chinju, South Korea*

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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

* Corresponding author. Tel./fax: +82 62 230 6639.
E-mail address: hgjeong@chosun.ac.kr (H.G. Jeong).
[1] The first two authors contributed equally to this work.

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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,