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Effect of *Prunella vulgaris* Labiatae Extract on Innate Immune Cells and Anti-metastatic Effect in Mice

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Abstract Ability of water extract from *Prunella vulgaris* Labiatae to stimulate immune system and inhibit tumor metastasis in mice was assessed. In experimental lung metastasis, prophylactic intravenous (i.v.) administration of water extract from *P. vulgaris* significantly inhibited lung metastasis in a dose-dependant manner. Peritoneal macrophages stimulated with *P. vulgaris* produced various cytokines such as tumor necrosis factor (TNF)-α and interlukin (IL)-12 as well as induced tumoricidal activity. In an assay for natural killer (NK) cell activity, i.v. administration of *P. vulgaris* significantly augmented NK cytotoxicity. The depletion of NK cells by injection of rabbit anti-asialo GM1 serum abolished the inhibitory effect of *P. vulgaris* on lung metastasis of colon26-M3.1 cells. These data demonstrate that *P. vulgaris* activate innate immune system to inhibit the growth of foreign materials including tumor cells in mice.

Keywords: *Prunella vulgaris* L., innate immunity, tumor metastasis, cytokine

Introduction

In experimental tumor metastasis model, natural product involving oriental medicines, cytokines, and synthetic adjuvants, have been thought to be a useful tool to suppress tumor growth and inhibit tumor metastasis (1-3). In fact, various natural products having biological activity to enhance host defense system have been applied to cancer immunotherapy (4-7) or one of the most attractive alternatives to cytotoxic drugs (8). Metastasis is one of the characteristics that distinguish malignant tumors from benign neoplasm (9). Progressive metastasis of tumor cells in the host results in a wide range of biological heterogeneity for immunogenicity, such as growth rate, cell markers, and sensitivity to chemotherapeutic drugs (9). Therefore, preventing metastasis is one of the most crucial issues in cancer treatment. It is well known that the activation of the innate immune system is thought to play a critical role of defense system against foreign antigens including tumors (10). The primary mechanisms of immunostimulating activities by natural products are due to their ability of activate macrophages or NK cells (1,3,10). Activated NK cell and macrophages can lyse or inhibit the growth of a wide variety of tumor cells (2,11,12). Indeed, many experimental studies and clinical trials showed that natural immunity played an important role in blocking of metastasis from primary tumors (2,10,12-14). In addition, various cytokines, such as interleukin (IL)-12, tumor necrosis factor (TNF)-α, or IL-1β from macrophages, are demonstrated to augment NK cell responses and these proinflammatory cytokines can induce activation of adaptive immunity in part through stimulation of interferon (IFN)-γ production from natural killer cells (4,15-17).

Prunella vulgaris is a perennial plant known for its self-heal in Western herbal medicine (18), and traditionally has been used in treating various diseases in East Asian countries such as an allergy and inflammation (19). In addition, *P. vulgaris* has been reported to have an immunomodulatory effect like activation of macrophages (20), only a little evidence has been established on the anti-tumor activity of *P. vulgaris* either *in vitro* or *in vivo* (18-20). Furthermore it is still unknown if *P. vulgaris* has anti-tumor metastasis activity by activation of innate immune system.

This study demonstrated that the water extract from *P. vulgaris* (PVL) inhibited tumor metastasis in animal model and its anti-tumor activity was associated with activation of innate immune cells such as macrophages and NK cells. To study the immunostimulating effect of PVL on tumor metastasis, the experimental tumor metastasis models were formed in syngeneic mice (BALB/c, C57BL/6, and CDF1) with colon26-M3.1 carcinoma, B16-BL6 melanoma, and L5178Y-ML25 lymphoma cells, respectively, followed by systemic administration of PVL. Then, the activities of NK cells and macrophages were analyzed to clarify its anti-metastatic effect on tumors.

Materials and Methods

Plant material and extraction *Prunella vulgaris* originating from South Korea were kindly donated by an herbal

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medicine company (Sanyacho-Nongwon, Namyangju, Korea). The chopped flowers stem and leaves of *P. vulgaris* (100 g) were placed in distilled water (1,000 mL) and stirred at 4°C overnight. After centrifugation at 15,000×g for 20 min, the supernatant was filtered with 0.2-μm pore sized filters. Protein content of *P. vulgaris* extract (called as PVL) was determined by using of commercial protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) and stored at 4°C until use.

Mice and cell cultures Specific pathogen-free female BALB/c, C57BL/6, and CDF1, 6 weeks old, were purchased from Jungang Lab Animal Inc. (Seoul, Korea). The mice were housed in a specific pathogen-free environment. Experiments were conducted in accordance with the guidelines established by the Animal Care and Use Committee of Yuhan University. Water and diet of pellets were supplied *ad libitum*. A lung metastatic subline of a highly metastatic cell line of colon 26 carcinoma, colon 26-M3.1 and a lung metastatic subline of the murine B16, B16-BL6 melanoma cells were maintained as monolayer cultures in Eagle's MEM supplemented with 7.5% fatal bovine serum (FBS), sodium pyruvate, non-essential amino acids, and L-glutamine which were purchased from Gibco BRL (Grand Island, NY, USA) as described previously (16,20,27). L5178Y-ML25 lymphoma cells and splenocyte from mice was cultured in RPMI-1640 (Gibco BRL) supplemented with 7.5% FBS and L-glutamine (17).

Cytotoxicity assay In cytotoxicity test, various doses of PVL (0.4-500 μg/mL) in culture medium were added into each well of the culture plate of B16-BL6 cells (1×10^4/well) or splenocytes (2×10^5/well) from normal mouse, and incubated for 72 hr. Cytotoxicity against these cells was assayed by a WST-1 based colorimetric assay (Wako Pure Chemicals Industries, Ltd., Osaka, Japan) (14). Absorbance of each well was monitored at 450 nm using a microtiter plate reader (Molecular Device, Sunnyvale, CA, USA).

Experimental lung metastasis Experimental lung metastasis were induced by i.v. inoculation of colon26-M3.1 carcinoma cells (3×10^4) and B16-BL6 melanoma cells (4×10^4) into BALB/c and C57BL6 mice, respectively (13). To study the anti-tumor metastasis activity of PVL, groups of 5 BALB/c mice were given i.v. administration of PVL (1-100 μg/mouse) 2 days before tumor cell inoculation. The mice were sacrificed 14 days after tumor inoculation and their lungs were fixed in Bouin's solution. Lung tumor colonies were counted microscopically. Liver and spleen metastasis were analyzed by i.v. inoculation of L5178Y-ML25 cells (4×10^4 cells) into CDF1 mice. The weight of liver and spleen was recorded to evaluate tumor metastasis at 14 days after tumor inoculation (17).

Cytokine assay Peritoneal macrophages were harvested from thioglycollate-treated mice as described previously (4). The cells (1.5×10^6/mL/well) suspended in complete RPMI-1640 medium were plated into 24-well culture plates (Nalge Nunc International, Roskilde, Denmark). After 2 hr incubation, non-adherent cells were removed by washing with phosphate buffered saline (PBS), the adherent macrophages were co-incubated with the indicated doses of PVL for 24 hr. The respective concentration of TNF-α and IL-12 in the culture supernatants was determined by enzyme-linked immunosorbent assay (ELISA) kits (Pharmingen Co., San Jose, CA, USA) according to the manufacturer's recommendations.

***In vitro* assay of macrophage-mediated cytotoxicity** In brief, colon26-M3.1 cell (1×10^4) were added to the macrophage monolayers harvested from the mice given intraperitoneal (i.p.) administration of PVL (100 μg/mouse) 3 days before the assay in 96-well plates to obtain various effector (macrophage)-to-target cell (colon26-M3.1) ratios as previously described (5). The cultures were incubated for 18 hr. After centrifugation, the culture supernatants were admixed with lactate dehydrogenase (LDH) solution (BioAssay Systems, Hayward, CA, USA), and the absorbance value of each well was measured at 490 nm using a microtiter plate reader (Molecular Device). The % macrophage cytotoxicity was calculated from the following formula: Cytotoxicity (%)=[(OD value of experimental group−OD value of spontaneous group)/(OD value of untreated group−OD value of spontaneous group)]×100.

Assay of NK-mediated tumor cytotoxicity Three BALB/c mice/group were administered i.v. with 100 μg of PVL, and the BALB/c splenocytes were harvested 2 days after PVL treatment. Single cell suspensions of the splenocytes were added to Yac-1 cells (1×10^4/well) to obtain effector (splenocytes)-to-target cell (YAC-1) ratio (E/T ratio) of 100:1, 50:1, or 25:1 in a U-bottomed 96-well plate, and the cultures were incubated for 6 hr. After centrifugation, the culture supernatants were admixed with LDH solution (BioAssay Systems), and the absorbance value of each well was measured at 490 nm. NK cell cytotoxicity was calculated with same method in assay of macrophage-mediated cytotoxicity.

Depletion of NK cells in mouse Depletion of NK cells *in vivo* was performed according to the method described previously (21). Mice were injected i.p. with 500 μL/mouse of 50-fold diluted rabbit anti-asialo GM1 serum (Wako) 1 and 3 days before tumor inoculation.

Statistical analysis The statistically significant difference between the groups was calculated by applying the Student's two-tailed *t* test.

Results and Discussion

Inhibitory effect of PVL on experimental lung metastasis To investigate whether PVL can enhance natural immunity against tumors, we have examined the effect of PVL on the experimental lung tumor metastasis of colon26-M3.1 and B16-BL6 melanoma cells. The prophylactic i.v. administration of PVL (1-100 μg/mouse) dependently inhibited lung metastasis of both tumor cell types. Administration of 100 μg of PVL inhibited lung metastasis by colon26-M3.1 carcinoma over 90% suggesting that the optimal dose to inhibit tumor metastasis in mice were 100 μg/mouse. This pattern was similar to other type of metastasis model

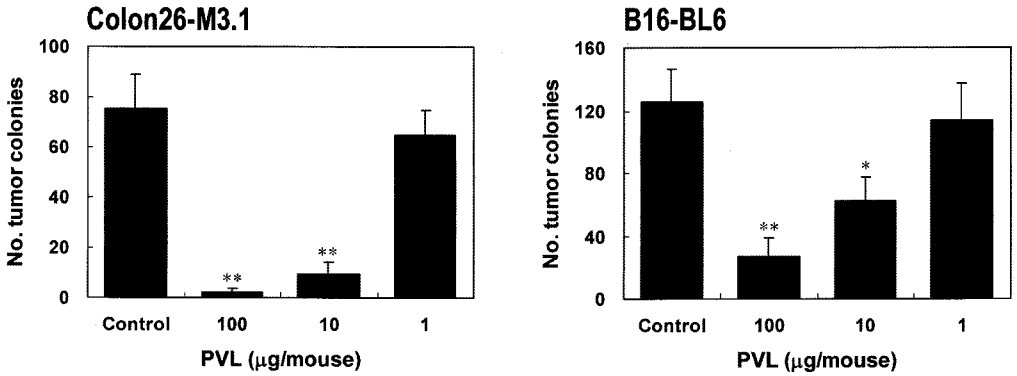

Fig. 1. Prophylactic effect of PVL on lung metastasis produced by i.v. inoculation of colon26-M3.1 carcinoma or B16-BL6 melanoma cells. *$p<0.01$, **$p<0.001$; compared with untreated control (by Student's two-tailed t-test).

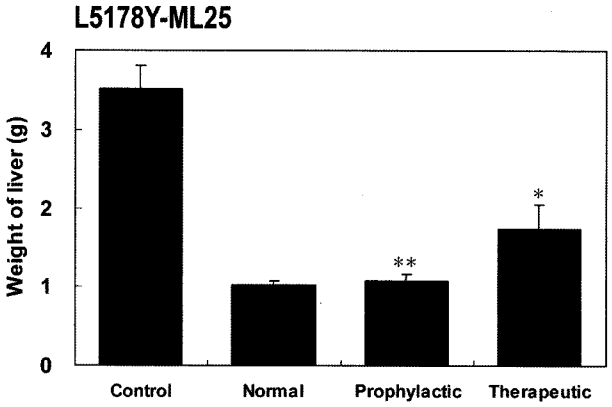

Fig. 2. Prophylactic or therapeutic effect of PVL on liver metastasis produced by i.v. inoculation of L5178Y-ML25 cells. *$p<0.01$, **$p<0.001$; compared with untreated control (by Student's two-tailed t-test).

produced by B16-BL6 melanoma (Fig. 1). In addition, the administration of PVL to mice dramatically inhibited liver metastasis of L5178Y-ML25 lymphoma cells (Fig. 2). The data obtained from our *in vivo* experiments indicated that i.v. administration of PVL resulted in the prophylactic or therapeutic inhibition of tumor metastasis produced by 3 types of tumor cells. The administration of PVL in those ranges did not show any apparent side effect such as a decrease of body weight or piloelection (data not shown).

Cytotoxicity assay Cytotoxic effect of PVL on tumor cells and murine splenocytes was examined in an *in vitro* experiment. As shown in Fig. 3, PVL at the concentration up to 100 μg/mL did not affect the growth of normal or tumor cells. These results indicate that the inhibitory activity of PVL on tumor metastasis in mice is not due to its cytotoxic effect on tumor cells. Numerous studies demonstrated that the inhibition of tumor metastasis by BRMs in animal model was associated with activation of innate immune cells such as macrophages and NK cells (2,3,11,12). We therefore examined whether PVL can stimulate innate immune system such as macrophages and NK-cells involved the anti-metastatic activity through them.

Activation of macrophages It is well known that activated macrophages release various cytokines (5-7,13), and that these cytokines play a role in inducing and modulating immune responses to elicit potent anti-tumor or anti-metastatic activities. We examined whether the treatment of PVL can activate macrophages with the result of cytokine production. As shown in Fig. 4, PVL treatment led peritoneal macrophages to induce various cytokines such as TNF-α and IL-12. Inflammatory cytokines like IL-1β and TNF- α, which are secreted from macrophages, play a role in activating T cells and rejecting tumor cells

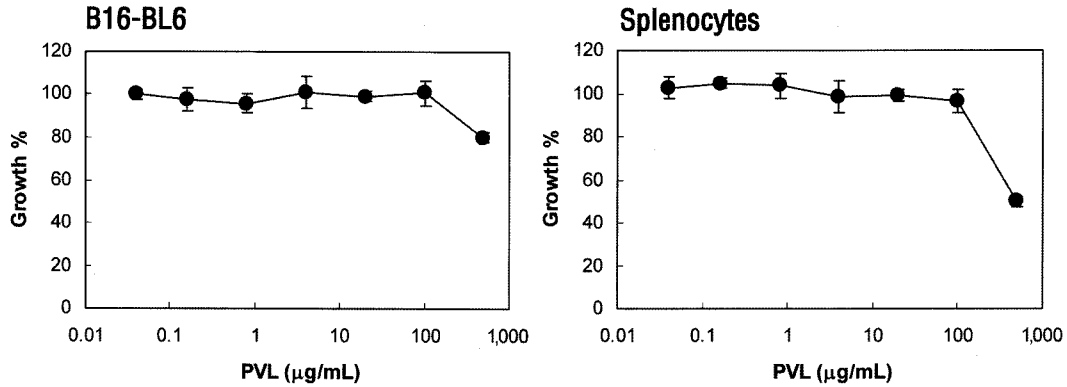

Fig. 3 Effect of PVL on the growth of tumor cells and normal splenocytes. B16-BL6 or the splenocytes were co-incubated with the indicated doses of PVL for 72 hr.

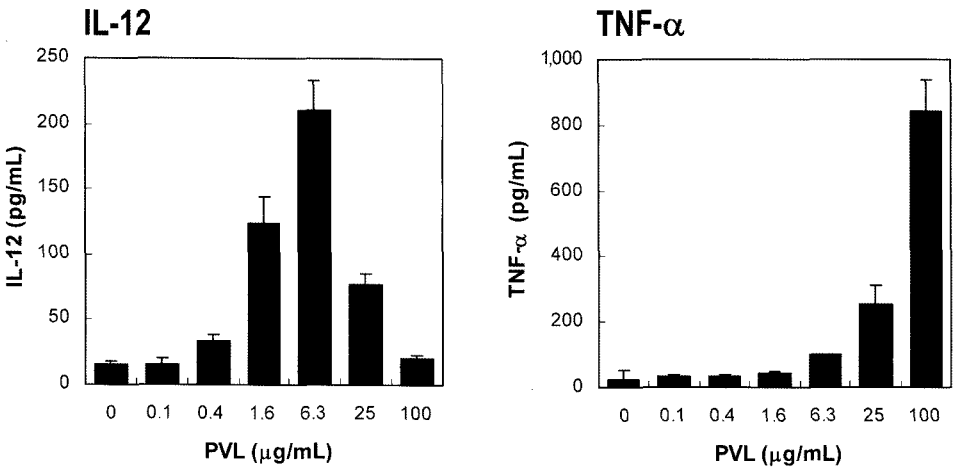

Fig. 4. Effect of PVL on induction of cytokines from macrophages. The level of cytokines in the group treated with LPS (5 mg/mL) was as follows. IL-12, 535.8±46.7; TNF-α, 204.5±76.4 pg/mL.

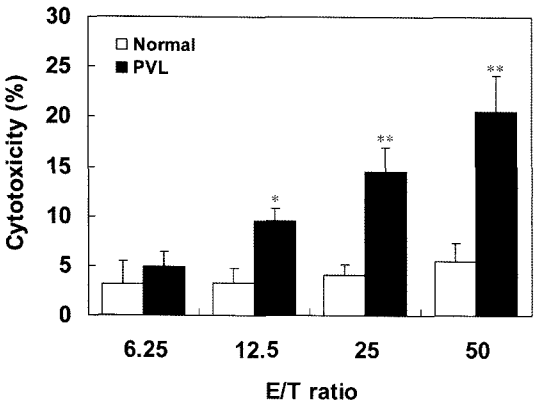

Fig. 5. Effect of PVL on macrophage-mediated cytotoxicity against tumor cells. *p<0.01, **p<0,001; compared with the untreated group (by Student's two-tailed t-test).

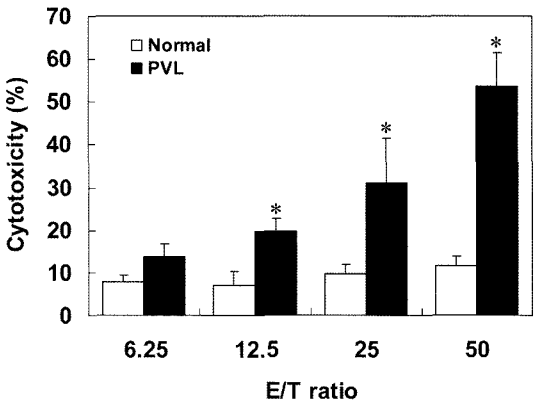

Fig. 6. Effect of PVL on the enhancement of NK cell activity. *p<0.01, **p<0,001; compared with the untreated group (by Student's two-tailed t-test).

(22,23). TNF-α is one of the cytokines involved in evoking immunoregulation and anti-tumor mechanisms (22,24). Therefore, TNF-α has been extensively tested in *in vitro* and *in vivo* investigations as well as in clinical trials for immunotherapy of malignant diseases. In addition, IL-12 is produced from phagocytic antigen-presenting cell such as macrophage and dendritic cells in early stages of the immune response (14). IL-12, which is known as multi-functional cytokine, is said to be one of the most essential cytokines to elicit tumor immunity (14,15,23). In addition, activated macrophages stimulated by IFN-γ and autologous IL-12 can eradicate tumor cells by releasing of TNF-α and nitric oxide *in vitro* (14,15,25). In fact, peritoneal macrophages obtained from PVL-treated mice displayed a higher cytolytic activity against tumor cells than those of the untreated mice (Fig. 5). The ability to induce cytokine release from macrophages and enhance macrophage-mediated cytotoxicity against tumor cells by treatment of PVL may be one of the major mechanisms related to the enhancement of host natural immunity in inhibiting tumor metastasis (5,6,12,13).

Effect of PVL on NK cell activity Activated NK cells are relevant effectors responsible for natural immunity against tumors (12,13,21). Many investigators have demonstrated that the activation of NK cells by immuno-stimulants led to reduction of metastatic colonization of tumors (5,21). Therefore, at the point of NK cell activation, we also analyzed the mechanism by which PVL can inhibit tumor metastasis. The effect of PVL on enhancing NK cell activity was analyzed by testing the cytotoxicity against Yac-1 cells. As shown in Fig. 6, the splenocytes obtained from mice administered i.v. with PVL 2 days before the assay showed a higher cytotoxicity than those of the untreated mice in an E/T ratio-dependent manner. Since NK cells are known to be an important effector to suppress tumor growth and metastasis (5,13,21,26), we next investigated whether the inhibitory effect of PVL (100 μg) on tumor metastasis was associated with the activated NK cell activity using an experimental metastasis model. As seen in Fig. 7, pretreatment with anti-asialo GM1 serum which can selectively eliminate NK cell enhanced the frequency of experimental lung metastasis as compared with that found in the untreated mice. The deletion of NK cells abolished the anti-tumor effect of PVL on lung metastasis of colon26-M3.1 cells, indicating that the

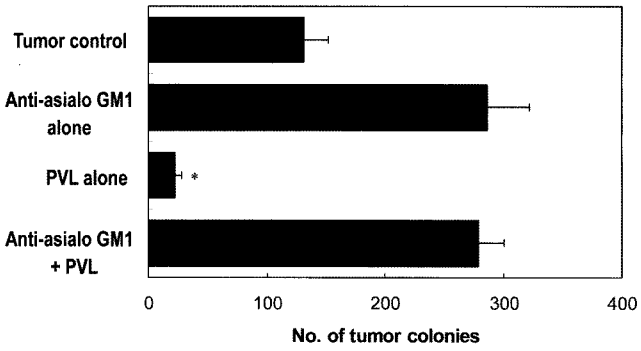

Fig. 7. Effect of NK cell depletion on PVL-induced inhibition of lung metastasis. *$p<0.001$, compared with the untreated group (by Student's two-tailed *t*-test).

inhibitory effect of PVL on tumor metastasis was partly mediated by NK cell activation (13,21).

In conclusion, PVL is likely to require co-activation of both NK cells and macrophages to obtain its anti-tumor activity and this is agreed with other results (2,6,7,13). Further study to elucidate active molecules and biological mechanisms related to anti-tumor activity of PVL is currently underway in our laboratory.

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