SPANDIDOS PUBLICATIONS

A ginseng saponin metabolite suppresses tumor necrosis factor-α-promoted metastasis by suppressing nuclear factor-κB signaling in murine colon cancer cells

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Abstract. SC-514, an inhibitor of IκB kinase ß (IKKß), blocked the TNF-α-induced activation of nuclear factor-κB (NF-κB) as well as the TNF-α-promoted metastasis of murine colon adenocarcinoma cells. We investigated the effect of 20-*O*-ß-D-glucopyranosyl-20(S)-protopanaxadiol (M1), a main intestinal bacterial metabolite of ginseng, on the NF-κB-dependent metastasis. M1 was effective in suppressing the TNF-α-induced activation of NF-κB, expression of matrix metalloprotease-9 (MMP-9), migration and invasion. The TNF-α-evoked increase in lung and liver metastasis of colon carcinoma was also abrogated by treatment with M1 *in vitro*. These results suggest that ginseng has potential to suppress inflammation-related metastasis by downregulating the NF-κB signaling pathway.

Introduction

Ginseng (the root of *Panax ginseng* C.A. Mayer, Araliaceae) has been used for thousands of years as a traditional medicine in many countries including Korea, Japan and P.R. China. Major components of ginseng are triterpene saponins, named ginsenosides, which are divided into two groups; protopanaxadiol-type (Rb1 and Rb2) and protopanaxatriol-type (Rg1) (1,2). Protopanaxadiol-type ginsenosides are metabolized by human intestinal bacteria to 20-*O*-ß-D-glucopyranosyl-20(S)-protopanaxadiol (M1, also known as compound K), which is then absorbed from the gastrointestinal tract (3-6). The metabolite has a variety of pharmacological activities including anti-tumor, anti-diabetic, anti-inflammatory and anti-allergic effects (7-14).

During the metastatic cascade, many pathogenic changes occur including inflammation. Tumor necrosis factor-α (TNF-α) plays a critical role in tumorigenesis by regulating a cascade of expression of the gene for cytokines, adhesion molecules, extracellular proteases, and pro-angiogenic molecules (15,16). We have established an *in vitro* TNF-α-stimulation model for cancer metastasis using colon 26 cells, and demonstrated that the enhancement of pulmonary metastasis is associated with activation of extracellular-signal regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinases (MAPKs) (17,18). However, NF-κB, another factor critical for TNF-α signaling, remains to be characterized.

We have demonstrated the anti-metastatic effects of ginseng using experimental metastasis models with B16-BL6 melanoma, colon 26 adenocarcinoma, and Lewis lung carcinoma (LLC) cells *in vivo* (7,8,19). The anti-tumor activities of ginseng are thought to be based on intestinal metabolites such as M1; however, the mechanisms of action of these metabolites remain to be fully elucidated. Therefore, we investigated the effect of M1 on inflammation-related metastasis, and found that M1 suppressed TNF-α-enhanced metastasis by downregulating NF-κB signaling in colon 26 cells.

Materials and methods

Reagents. Recombinant human TNF-α was purchased from GenzymeTECHNE (USA). Antibodies against phospho-NF-κB p65 (Ser^{536}) and p65(C-20) were obtained from Cell Signaling Technology (Beverly, MA) and Santa Cruz Biotechnologies (CA, USA), respectively. SC-514, a selective IKKß inhibitor, was purchased from Calbiochem (Germany).

Isolation of M1. M1 (compound K) was isolated from ginseng as described previously (14). Briefly, ginseng (KyungDong Market, Seoul, Korea) was extracted with MeOH and fractionized with BuOH. The BuOH extract was transformed with *Fusobacterium* K-60, a human intestinal bacterium. 20-*O*-ß-D-glucopyranosyl-20(S)-protopanaxadiol (M1,

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Abbreviations: TNF-α, tumor necrosis factor-α; NF-κB, nuclear factor-κB; IκBα, inhibitor κBα; IKK, IκB kinase; MMP, matrix metalloproteinase; MAPK, mitogen-activated protein kinase; ERK, extracellular-signal regulated kinase; JNK, c-Jun N-terminal kinase

Key words: TNF-α, NF-κB, metastasis, ginseng

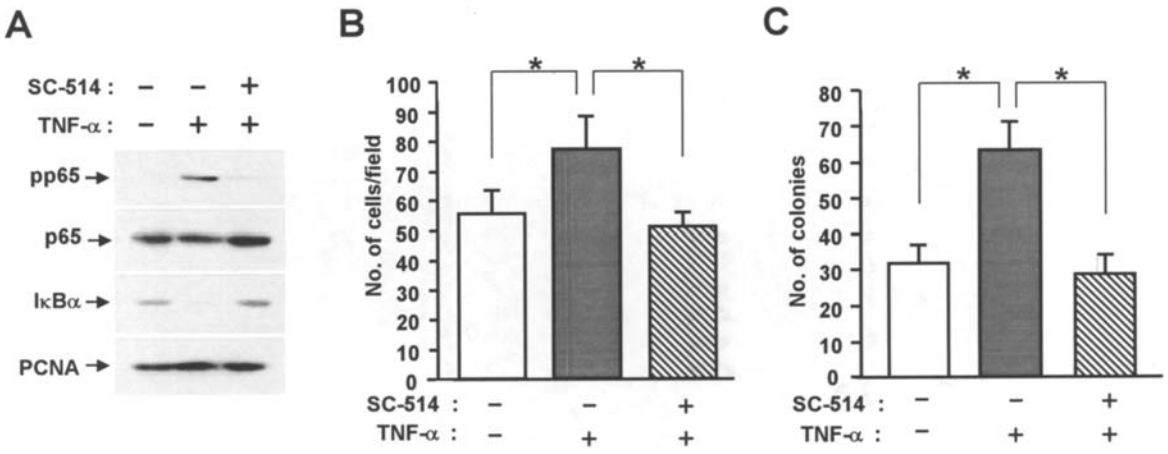

Figure 1. Suppression of TNF-α-promoted metastasis by SC-514. (A) Colon 26 cells were pretreated with SC-514 (100 μM) for 30 min, and then stimulated with TNF-α (10 ng/ml) for 10 min. The cell lysates were immunoblotted with antibodies against IκBα, phospho-p65, p65, and PCNA. (B) Cells were treated with SC-514, and then stimulated with TNF-α for 6 h. The cell suspension ($1x10^4$/100 μl) was added to the upper chamber which was precoated with fibronectin (1 μg) on its lower surface. After 4 h of incubation, the filters were stained with hematoxylin and eosin, and the cells that had migrated onto the lower surface were counted in five predetermined fields. Data are represented as the mean ± S.D. of triplicate experiments. *p<0.01. (C) Cells were treated with SC-514, and then stimulated with TNF-α for 6 h. The cell suspension ($3x10^4$/200 μl) was inoculated intravenously. On day 14, the mice were sacrificed and the tumor colonies in the lung were enumerated to evaluate lung metastasis. Data are presented as the mean ± S.E. for seven mice in each group. *p<0.01.

compound K) was isolated by silica gel column chromatography using $CHCl_3$-MeOH-H_2O (10:3:1, lower layer).

Animals. Six-week-old specific pathogen-free female BALB/c mice were purchased from Japan SLC (Hamamatsu, Japan). The mice were maintained in the Laboratory for Animal Experiments, Institute for Natural Medicine, University of Toyama, under laminar air flow conditions with a 12-h light/dark cycle at a temperature of 22-25˚C. The mice were used according to institutional guidelines.

Cell culture and treatment. Colon 26 cells were maintained in RMPI-1640 medium (Gibco BRL, Life Technologies Inc., NY, USA) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 100 units/ml of penicillin and 100 μg/ml of streptomycin. Cultures were kept at 37˚C in a humidified atmosphere of 5% CO_2/ 95% air.

Colon 26 cells were stimulated with recombinant human TNF-α at a concentration of 10 ng/ml for 6 h *in vitro* and then used for *in vitro* assays and *in vivo* models. M1 and SC-514 (100 μM) were added for 30 min before TNF-α.

Western blotting. Whole cell lysates from colon 26 cells were prepared with lysis buffer [25 mM HEPES pH 7.7, 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton X-100, 20 mM ß-glycerophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), 10 μg/ml aprotinin and 10 μg/ml leupeptin]. Cell lysates were resolved by SDS-PAGE (10%) and transferred to an Immobilon-P nylon membrane (Millipore, MA, USA). The membrane was treated with BlockAce (Dainippon pharmaceutical Co. Ltd, Suita, Japan) overnight at 4˚C and probed with anti-phospho-NF-κB p65(Ser^{536}) and anti-p65(C-20) antibodies. The primary antibody was detected using horseradish peroxidase-conjugated anti-rabbit IgG (Dako, Denmark) and visualized with the ECL system (Amersham Biosciences, UK).

Migration and invasion assays. The migration and invasion assays of colon 26 cells were performed in Transwell cell culture chambers according to methods reported previously (20). In the migration assay, the filters were precoated with 1 μg of fibronectin on the upper surface, while in the invasion assay, they were coated with 1 μg of fibronectin and Matrigel on the lower and upper surfaces, respectively. The cell suspension ($2x10^4$/100 μl) was added to the upper compartment and incubated for 4 h (migration) or 6 h (invasion). The migrated or invaded cells were counted under the microscope in five predetermined fields at a magnification of x400. Each assay was performed in triplicate.

In vivo metastasis models. Colon 26 cells were harvested with trypsin-EDTA, washed with serum-free RPMI-1640 medium, and resuspended in cold PBS. In the lung metastasis model, the cell suspension ($3x10^4$/200 μl) was implanted by intravenous injection. The mice were sacrificed on day 14 after tumor inoculation and the lungs were removed. The metastatic tumor colonies in the lungs were enumerated microscopically. To metastasize to liver, the cell suspension ($2x10^4$/200 μl) was injected into the portal vein of mice which had been anesthetized with ether and laparotomized. Eighteen days after the tumor inoculation, the mice were sacrificed and the livers were removed. Liver weight was recorded to evaluate tumor metastasis as described previously (21,22).

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA from colon 26 cells was isolated using an RNeasy mini kit (Qiagen, CA, USA) according to the manufacturer's instructions. First-strand cDNA was synthesized using an oligo$(dT)_{18}$ primer and Superscript II reverse transcriptase (Invitrogen Life Technologies Inc., CA, USA). The reaction profile was 42˚C for 50 min, followed by 70˚C for 15 min. PCR amplification of the cDNAs was performed with denaturation at 95˚C for 30 sec, annealing at 60˚C for 30 sec and extension

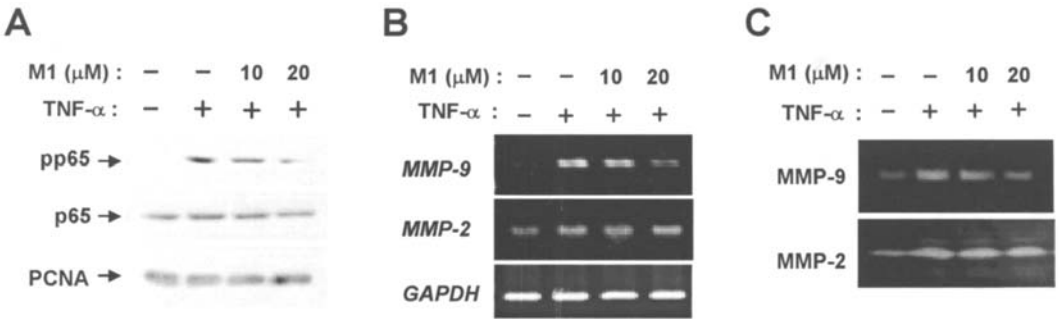

Figure 2. M1 suppressed TNF-α-induced NF-κB activation and MMP-9 expression. (A) Colon 26 cells were treated with M1 (10, 20 nM) and then stimulated with TNF-α for 10 min (A) and 6 h (B and C). Immunoblotting assays of phospho-p65, p65 and PCNA were performed as described above. (B) Total RNA from cells was isolated and first-strand cDNA was synthesized. The PCR amplification was performed using primers described in the Materials and methods. (C) MMP-9 enzymatic activity was determined by gelatin zymography using the conditioned media. MMP-9 activity was expressed in relation to the untreated group. Similar results were obtained in three independent experiments.

at 72˚C for 1 min, using an Ex Taq PCR kit (Takara Shuzo Co. Ltd., Shiga, Japan) with specific primers as follows: MMP-9, 5'-TTCTCTGGACGTCAAATGTGG-3' and 5'-CAAAGAAGGAGCCCTAGTTCAAGG-3'; MMP-2, 5'-CCTGATGTCCAGCAAGTAGATGC-3' and 5'-TTAAGGT GGTGCAGGTATCTGG-3'; and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 5'-GGTGAAGGTCGGTGTGAA CGGATTT-3' and 5'-CATGCCAAAGTTGTCATGGATGA CC-3'. The PCR products were electrophoresed on 1% agarose gels and detected by ethidium bromide staining.

Gelatin zymography. Gelatin zymography was performed as described with some modifications (21). The conditioned medium was concentrated using Centricon (Millipore, MA, USA) according to the manufacturer's instructions and was electrophoresed at 4˚C on a 7.5% SDS-polyacrylamide gel containing 0.1% gelatin. After the electrophoresis, the gel was rinsed twice in 50 mM Tris-HCl, containing 2.5% Triton X-100, 5 mM $CaCl_2$, 1 mM $ZnCl_2$, and 0.05% NaN_3 to remove the SDS, and incubated for 24 h at 37˚C in incubation buffer (50 mM Tris-HCl containing 5 mM $CaCl_2$, 1 mM $ZnCl_2$, and 0.05% NaN_3). The gel was stained with a Coomassie brilliant blue solution (0.1% Coomassie brilliant blue, 10% acetic acid, and 10% isopropanol) and destained with destaining solution (10% acetic acid with 10% isopropanol). Photographs were taken using the Chemi-Doc XRS system (Bio-Rad, USA)

Statistical analysis. The significance of differences between groups was determined by applying the Student's two-tailed t-test.

Results

Role of NF-κB signaling in TNF-α-promoted metastasis. We first confirmed the TNF-α-induced activation of the NF-κB signaling pathway, activation of IKK complex, degradation of IκBα and phosphorylation of NF-κB p65 at Ser-536, in colon 26 cells (data not shown). SC-514, a selective IKKß inhibitor, blocked the degradation of IκBα and phosphorylation of p65 (Fig. 1B), demonstrating that TNF-α induced activation of the canonical NF-κB pathway. In order to investigate the role of NF-κB signaling in the TNF-α-enhanced metastasis, we next examined the effects of SC-514 on metastasis. Suppression of the signaling pathway abrogated the TNF-α-enhanced cell migration *in vitro* (Fig. 1C). Moreover, TNF-α-promoted pulmonary metastasis was also abrogated by SC-514 (Fig. 1D). A similar result was obtained in experimental metastasis to the liver (data not shown). These results clearly indicate that the NF-κB p65 signaling pathway is involved in TNF-α-promoted metastasis.

Effect of ginsenoside M1 on TNF-α-induced metastatic properties in vitro. We investigated the effect of M1, a main intestinal bacterial metabolite of ginseng saponins, on the TNF-α-enhanced metastatic properties of colon 26 cells. M1 inhibited partially the TNF-α-induced activation of NF-κB in a concentration-dependent manner (Fig. 2A). M1 is reported to inhibit the secretion of MMP-9 induced by several stimuli including PMA. RT-PCR confirmed a similar inhibitory effect of M1 on the TNF-α-induced expression of MMP-9 mRNA in colon 26 cells (Fig. 2B). In contrast, there was no obvious inhibition of the slight increase in MMP-2 mRNA expression induced by TNF-α (Fig. 2B) and the constitutive expression of TIMP-1, and 2 mRNAs (data not shown). Similarly, gelatin zymography demonstrated that M1 inhibited the increased enzyme activity of MMP-9 in a concentration-dependent manner without affecting MMP-2 activity (Fig. 2C). These results demonstrated that M1 selectively inhibited MMP-9 by downregulating the activation of NF-κB.

M1 inhibited TNF-α-promoted migration and invasion. We have previously demonstrated that some ginseng metabolites inhibited migration and invasion in several tumor cell lines. Here, we investigated the effect of M1 on the TNF-α-enhanced migration and invasion of colon 26 cells. After a 30-min pretreatment with M1, cells were stimulated with TNF-α for 6 h, and then subjected to migration and invasion assays. There was no significant cytotoxicity among tumor cells under these conditions (data not shown). Fig. 3A shows that M1 suppressed the TNF-α-promoted migration (Fig. 3A) and invasion (Fig. 3B) in a similar concentration-dependent manner.

M1 suppressed the TNF-α-enhanced metastasis. Finally, we investigated the effect of M1 on the TNF-α-promoted metastasis *in vivo*. Cultured colon 26 cells were treated with M1 at 20 μM, and then stimulated with TNF-α for 6 h *in vitro*. In

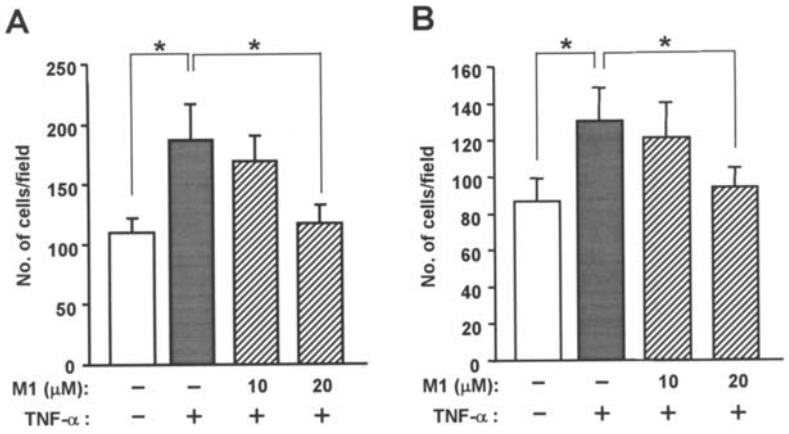

Figure 3. M1 suppressed TNF-α-promoted migration and invasion. Colon 26 cells were treated with M1 (10, 20 nM), followed by TNF-α for 6 h. (A) A migration assay was performed as described above. Data are represented as the mean ± S.D. of triplicate experiments. *p<0.01. (B) The cell suspension ($1x10^4$/100 μl) was added to the upper chamber which was precoated with fibronectin (1 μg) and matrigel (1 μg) on its lower and upper surface. After 6 h of incubation, the cells that had migrated onto the lower surface were counted in five predetermined fields. Data are presented as the mean ± S.D. for triplicate experiments. *p<0.01.

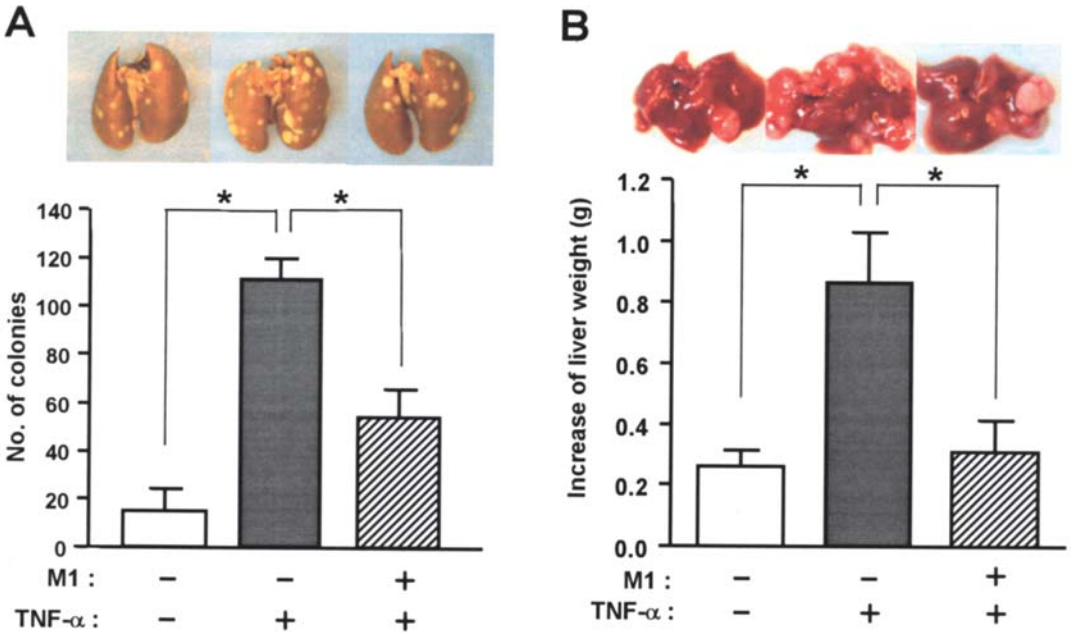

Figure 4. M1 abrogated TNF-α-promoted liver and lung metastasis. Colon 26 cells were treated with M1 (20 nM), followed by TNF-α for 6 h. (A) Pulmonary metastasis was examined as described above. Data are presented as the mean ± S.E. for seven mice in each group. *p<0.01. (B) The cell suspension ($2x10^4$/200 μl) was injected into the portal vein. Eighteen days after the inoculation, the mice were sacrificed and the increase in liver weight was measured to evaluate tumor metastasis. Data are presented as the mean ± S.E. for ten mice. *p<0.01.

models of metastasis to the lung or liver, the cells were inoculated via a tail vein or intraportal vein, respectively, and the number of colonies in the lungs or the increase of liver weight was determined on day 14 or 18 after the inoculation (Fig. 4). The TNF-α-induced increase in metastatic tumor colonies was significantly reversed by M1 (Fig. 4).

Discussion

We have already demonstrated that ginseng saponins significantly suppressed the metastatic properties of tumor cells and suggested that the action was based on their intestinal bacterial metabolites after oral administration (7,8,19). Lee *et al* reported that M1 had anti-inflammatory effects by inhibiting phorbol ester-induced COX-2 expression, which might contribute to antitumor effects on mouse skin carcinogenesis (23). However, it remains unclear whether or not M1 has an inhibitory effect in inflammation-associated cancer metastasis. Therefore, we investigated the effect of M1 on the TNF-α-enhanced metastasis of colon 26 cells using an *in vitro* TNF-α-stimulation model.

We determined the influence of M1 on the TNF-α induced signaling pathway. M1 is recognized to enhance the apoptotic cascade as well as to downregulate NF-κB signaling in many cancer cells (11,24). We also demonstrated that M1 suppressed the TNF-α-induced activation of the NF-κB p65 signaling

in colon 26 cells. However, treatment with M1 in ıce of TNF-α for 24 h did not induce the cleavage of caspase-3 and PARP (data not shown). This suggests that the inhibitory effect of M1 on the TNF-α-enhanced metastasis is not dependent on cell death, even though M1 suppressed the anti-apoptotic NF-κB pathway.

To confirm the inhibitory effect of M1 on the TNF-α-promoted metastasis, we investigated the expression of metastasis related genes. M1 was reported to suppress the expression and activity of MMP-9, which is usually regulated by NF-κB (25,26). In TNF-α-treated colon 26 cells, M1 suppressed the TNF-α-induced expression of MMP-9 at the level of both mRNA and protein. Jung *et al* also reported that M1 inhibited MMP-9 in human astroglioma cells, but they suggested that the inhibition was mediated through suppression of AP-1 (27). Besides M1, ginsenoside Rg3 was reported to reduce the gelatinolytic activities of MMP-9 and MMP-2 (28). We also determined the effect of other ginsenosides and their metabolites such as Rb1, Rg1, Rg3, Rh1 and Rh2, on the mRNA expression of MMP-9 induced by TNF-α, but none were effective in inhibiting the expression (data not shown). Therefore, M1 is thought to be an active metabolite of ginseng saponins with the potential to suppress inflammation-related metastasis.

Besides numerous reports about the effect of ginseng saponins on tumor cells, many investigators have reported the influence of ginseng saponins in the immune system. Nakaya *et al* reported that the production of TNF-α and IFN-γ was induced by ginseng saponins in spleen cells and peritoneal macrophages from C3H/HeN mice but not C3H/HeJ mice (29). Ginsan, a polysaccharide extract, markedly downregulated the production of proinflammatory cytokines (30). We also investigated the effect of M1 on the production of proinflammatory cytokines in peritoneal macrophage and spleen cells, and found that M1 suppressed the production of TNF-α in both cells (data not shown). These findings suggest that M1 has potential to suppress the inflammation-associated cancer growth by affecting both cancer cells and immune cells.

In summary, we focused on the effect of M1, a main metabolite of ginseng saponins, on inflammation-related metastasis, and found that the inhibitory effect of M1 on the TNF-α-promoted metastatic properties of colon 26 cells is related to down-regulation of NF-κB signaling. The cytokine stimulation model applied in this study might facilitate the evaluation of compounds effective in *in vivo* experimental models.

Acknowledgements

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