Inhibitory Effect of Agaricus Mixed Prescription on Metastasis and Tumor Formation

Ji Hee Chu, Nak Cheol Lim[1], Sung Hoon Kim*, Ki Won Ryu[2]

Department of Oncology, Graduate School of East-West Medical Science, Kyunghee University, 1: College of Oriental Medicine, Daejon University, 2: Oriental Medical College, Kyunghee University

Antimetastatic effects of Agaricus mixed prescription (AMP) were studied in the respect of blood-borne metastasis. For this aim, cytotoxicity against various cancer cells and normal cells, Chicken Chorioallantoic membrane (CAM) assay, cancer cell adhesion assay, platelet aggregation assay, pulmonary colonization, life span of S-180 implanted mice, and cytokine release assay were evaluated, respectively. The results were summarized as follows; AMP did not exert any cytotoxicity against all cell lines with IC50 of 25mg/ml on B16BL6. AMP disrupted formation of CAM at 1mg/ml. AMP was suppressive in adhesion assay of B16BL6. AMP also inhibited tumor induced platelet aggregation. In pulmonary colonization assay by B16BL6, the number of colonies in the lungs was significantly decreased in sample group than in control group. In animal study with S-180, the life span of AMP treated group was extended than that of control group. IL-12 was effectively increased in AMP treated group in cytokine release assay. Taken together, AMP can be possibly applied to cancer or metastasis.

Key words : Antimetastatic effect, Agaricus Mixed Prescription(AMP), CAM, Platelet aggregation, Pulmonary colonization, Cytokine

Introduction

Metastasis, the spread of malignant cells from a primary neoplasm to distant organs that results in the development of secondary tumors, is the most distinctive feature of malignant tumor. Advances in surgical techniques and adjuvant therapies have been proven to be useful in the treatment of primary tumors. However, metastasis remains a major cause of poor prognosis and death in cancer patients[1]. Surprisingly, several potent inhibitors are derived from tumors themselves. It was reported that angiostatin and endostatin which were secreted from the primary tumor prevented growth of metastases at the target organs[2-6], resulting that the removal of primary tumor by surgery caused the rapid growth of cancer.

Recently anticancer, anti-metastastic and anti-angiogenic agents were studied from natural products and oriental prescriptions, suggesting that those can be potently better drug with less side effects. We formulated AMP consisting of six herbs, Atroctylodes macrocephala, Polygonim multiflorum, Lycium Chinense, Gonoderma lucidum, Agaricus blazei.

* To whom correspondence should be addressed at : Sung Hoon Kim, Department of Oncology, Graduate School of East-West Medical Science, Kyunghee University, 1 Seochunri, Kiheungeup, Yongin 449-701, Korea,
· E-mail: sungkim7@khu.ac.kr, Tel: 031-201-2179

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Keum et al reported the methanol extract of heat-processed ginseng had antioxidant and antitumor promoting activities[7]. Moon et al reported panaxydol isolated from Panax ginseng induced G(1) cell cycle arrest and p27(KIP1) increase[8]. Mori et al reported the aqueous extracts from Atractylodes macrocephala had antitumor activity by immunopharmacological properties[9]. Zee-Cheng reported the Shi-quan-da-bu-tang, which is contained with Panax ginseng and Atractylodes macrocephala, had a potent biological response modifier in cancer immunotherapy, potentiation and detoxification of anticancer drugs[10]. Horikawa et al reported that Polygonum multiflorum inhibited the mutagenicity and carcinogenicity of benzo[a]pyrene, 1, 6-dinitroprene and 3,9-dinitrofluoranthene[11]. Cao et al reported that LAK/IL-2 plus Lycium barbarum polysaccharides (LBP) treatment led to marked increase in NK and LAK cell activity than LAK/IL-2 without LBP[12]. Wang et al reported the anti-tumor effect of Ganoderma lucidum[13] and Su et al reported that Ganodermic acid S, isolated from Ganoderma lucidum, exhibits inhibitory effects on platelet aggregation[14]. The Basidiomycete fungus Agaricus blazei MURILL in the prescription has traditionally been used as a health food for the prevention of cancer, diabetes, hyperlipidemia, arteriosclerosis and chronic hepatitis. The products from A. blazei MURILL have been studied about antitumor effect. Menoli et al reported antimutagenic effects of

Agaicus blazei extract on V79 cells[15]. Takaku et al reported that tumor growth was retarded by the oral administration of the lipid fraction extracted from A. blazei[16].

From above data, it was expected that AMP could be a potent prescription exerting antitumor and antimetastatic effects. Thus we evaluated the antimetastatic activity by way of CAM assay, adhesion assay, platelet aggregation assay, experiment. Moreover, immune activity of AMP was estimated, on the assumption that tumor cells must overcome host immune cell killing at all stage of metastasis

Materials and Methods

1. Prescription of AMP

AMP consists of six herbs as follows:

Crude drugs	Botanical Origin	Dose(g)
Ginseng Radix	Root of Panax ginseng C.A. MEY.	6
Atractylodis Macrocephalae Rhizoma	Rhizome of Atroctylodes macrocephala KOIDZ. or A. japonica KOIDZ	4
Polygoni Multiflori Radix	Bulb of Polygonim multiflorum THUNB.	4
Lycii Fructus	Fruit of Lycium Chinense MILL. or L. barbarum L.	4
Ganoderma	Fruit body of Gonoderma lucidum (LEYSS. ex FR.) KARST. or G. japonicum(FR.) LLOYD	4
Agaricus	Fruit body of Agaricus blazei MURILL	4
Total		26

2. Water Extract of AMP

Water Extract of AMP was prepared according to the method described elsewhere[17]. Briefly, AMP was placed in approximately 2,000ml of distilled water in round flask and boiled for 2 hrs using condense device. The water extract of AMP was filtered with Whatman filter (Cat No. 1002-300) and then concentrated with rotary vacuum evaporator (Eyela, Japan). This flask was placed in -84℃ deep freezer (Sanyo, Japan) for 24 hr and then dried by freeze dryer(Eyela, Japan) for 12hr. Yield(%) was 38%. An appropriate amount of the powder obtained was dissolved with PBS and filtered with 0.2(m (Lot No. 16534-000372). The solution was stored at 4 (C until use.

3. Cell lines

B16BL6 cells, a highly lung metastatic cell line of murine B16 melanoma, were maintained as monolayer cultures in minimum essential medium (MEM) supplemented with 10% heat inactivated fetal bovine serum (FBS, Gibco BRL, Cat No. 26140-079 U.S.A), non-essential amino acids, antibiotic-antimycotic (Gibco BRL, Cat No. 15240-062 U.S.A) and L-glutamine under a humidified atmosphere of 5% CO_2 in air as described previously[18]. Sarcoma-180 (S-180) cells were cultured in RPMI-1640 medium supplemented with 10% FBS, antibiotic-antimycotic and L-glutamone under a humidified atmosphere of 5% CO_2 in air.

4. Mice

Male C57BL/6J, Balb/C, and ICR mice were purchased from Dae Han Biolink. All mice were used at 4-6 weeks of age. They were maintained food and water ad libitum, and kept in a 12-hour light and dark cycle.

5. Cytotoxicity assay

MTT assay was done as previously described[19]. The principle of the MTT assay was based on the reduction of MTT to formazan by living but not by dead cells. The tetrazolium ring of MTT is cleaved by active mitochondrial dehydrogenase to produce brown formation precipitates. The solid residue was dissolved in DMSO. Cells were cultured in 96-well microtiter plates at 2 (10^4 cell per well in culture medium for 24hors. Each well was washed with PBS and then filled with 100ul free media containing sample (serial dilutions). Another 24hours later, cells were counted by MTT assay (Sigma, M-2128, U.S.A). Finally, the products were evaluated with an ELISA microplate reader (Molecular Devices, E-max precision microplate reader, S/N E10561, USA) at 570 nm. The concentrations of drugs giving 50% growth inhibition (IC50) were determined from experiments.

6. Chicken embryo chorioallantoic membrane (CAM) assay

The in vivo CAM angiogenesis model was used with minor modifications[20]. Fertilized eggs were incubated for 3days at 37℃, when albumin (about 3-5ml) was extracted from the air sac of egg. Two days after, a window was opened on the egg shell to expose the CAM. The window was covered with tape and the eggs were returned to the incubator until day 7. AMP containing various concentrations in a final volume of 10 ul of PBS on the thermanox coverslip was added to 9 day old CAMs at day 9 of chicken embryo development of the CAM, while ,in control group of eggs, only phosphate-buffered saline (PBS) was applied. The following day, intralipose (fat emulsion, 1ml) was injected below the CAM, and inhibition of angiogenesis was examined with the unaided eye.

7. Cell adhesion assay

ELISA 96 well plate was coated with Gelatin at 4℃ for 12 hours. Plate was washed three times with PBS and added with Bovine Serum Albumin (BSA, 10mg/ml) at 37℃ for 1 hour and washed three times with PBS again. The cells (5×10^4 cells/well) were collected with 0.1% trypsin-EDTA, and were

suspended in serum-free media containing AMP and plated into ELISA 96 well plate coated with Gelatin. Cell attachment was measured after incubation for 90 minutes at 37℃. The medium was removed and the well was rinsed twice with PBS. The attached cells on the well were stained with 0.5% crystal violet (50 ul) for 10 minutes and washed with tap water and then dried. Staining solution was resolved with acetic acid and measured OD value with ELISA reader at 550 nm. Data were expressed as a percentage.

8. Tumor cell induced platelet aggregation (TCIPA) assay

Platelet aggregation assay was performed in the blood samples collected from healthy subjects. Platelet-rich plasma (PRP) was obtained by centrifugation at 1,000rpm for 30 min followed by centrifugation at 12,000rpm for 10min to obtain platelet-poor plasma (PPP). PRP was diluted with PBS three fold. Platelet aggregation was carried out at 37℃ using an aggregometer (Chrono-log, U.S.A.), according to Born's method, as previously described[21]. Samples were added into 200ul PRP and the mixture was preincubated for 20min at 37℃. 300ul of B16BL6 cells (5×10^6 cells/ml) were added to the preincubated mixture. TCIPA was monitored by light transmission.

9. Experimental lung metastasis

Experimental lung metastasis of B16BL6 murine melanoma cells were assessed by intravenously (i.v.) inoculation of tumor cells into syngeneic C57BL/6 Jena mice [22]. Mice were inoculated with B16BL6 cells from 2 days after AMP treatment. AMP was administered orally with 10mg per mouse. The melanoma cells were collected from monolayer cultures by a brief trypsinization. The viability of the cells was determined with trypan blue, and a single cell suspension was made in PBS. Each mouse was injected via the lateral tail vein with 7×10^4 viable cells in 0.2mL PBS. To avoid possible changes in cell viability, melanoma cells were injected into mice within 30 min after their collection. The mice were killed 14 days after tumor inoculation and their lungs were separated and fixed in Bouin's solution. Lung tumor colonies were counted under a dissecting microscope.

10. Serum cytokine assay

AMP was orally treated to Balb/C mice in vivo three times once per two days. The next day, the mice were sacrificed to take blood. Blood was centrifuged at 2,500rpm for 10minutes. The supernatant was directly used for cytokine assay. 50 (l of standards or samples were added in duplicate to each well of the precoated strip well plate being utilized and reaction mixture was incubated at room temperature (20-25 ℃) for 1 hour and then wash the palte three times. Add 100 (l of biotinylated antibody reagent. Incubate the covered plate at room temperature for 1 hour. Wash the plate three times again. Dilute streptavidin-HRP concentrate in dilution buffer and add 100 (l of this solution to each well. Incubate the covered plate at room temperature for 30 minutes. Wash the plate three times. Add 100 (l premixed TMB substrate solution to each well. Develop the plate at room temperature for 30 minutes. Stop reaction by adding 100 (l of the provided stop solution to each well. Read the absorbance of the plate on a plate reader set at 450 minus 550 nm and calculate results using curve fitting statistical software.

11. Mean Survival Time in S-180 implanted mice

Measurement of mean span time was assessed by s.c. inoculation of S-180 cells into ICR mice as described previously [23]. Mice were inoculated with S-180 cells in 2 days after AMP treatment. AMP was administered orally with 0.2mL volume per mouse. The sarcoma cells were collected by a brief trypsinization. The viability of the cells was determined with trypan blue, and a single cell suspension was made in PBS. Each mouse was injected by s.c.with 3×10^6 viable cells in 0.2mL. To avoid possible changes in cell viability, sarcoma cells were injected into mice randomly within 10 min after their collection. AMP was administered everyday till the first death of mouse. The weight and death of mice were checked everyday.

12. Statistical analysis

Results are expressed as mean±S.D of control. The statistical significance of differences between the groups was determined by applying Student's t-test.

RESULTS

1. Cytotoxicity of AMP

To investigate cytotoxicity of AMP, MTT assay was used. Before AMP was treatment, cell (2×10^5 cell/ml) was prepared in 96-well microtiter plates. Sample or medium were added to cell plates for 24h with serum free medium. Cells were counted by MTT assay. O.D. value of cells was converted to % of control. The IC50 of AMP on B16BL6 was determined at a concentration of approximately 25mg/ml (Fig. 1. b). This finding indicates that cytotoxicity of AMP extremely low and AMP does not directly kill the metastatic cell. Besides B16BL6 cell lines, we evaluated the cytotoxic activity of different kinds of cancer cells (Fig. 1. c~d), these results are similar to B16BL6

melanoma cell. This means that AMP does not have toxicity for cancer cells.

The viability of normal cells exposed to various concentrations of AMP was also determined by MTT assays as reported previously. The viability of normal cells are very high, in spite of exposure to high concentration of AMP (Fig.1.e~ f). That is to say, there was no significant toxic effect on normal cells.

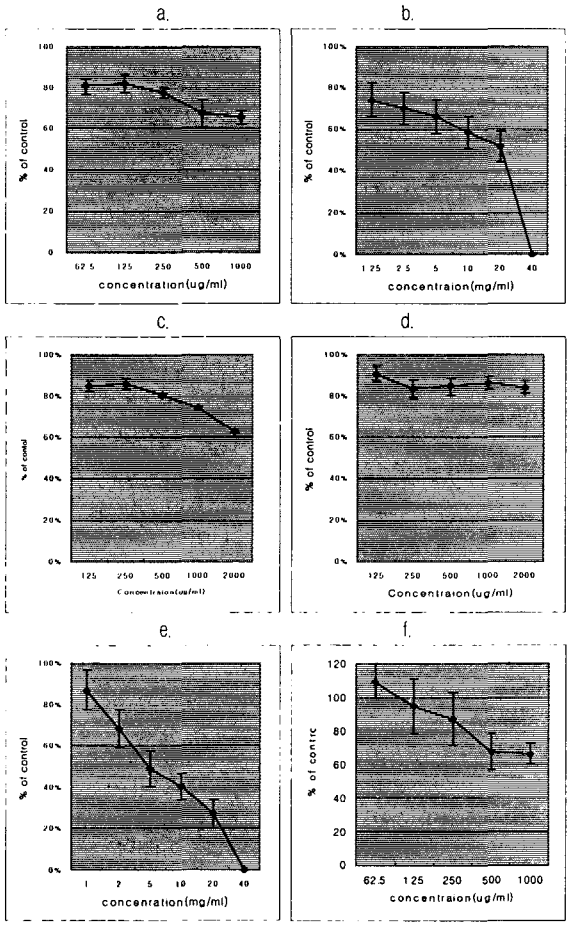

Fig. 1. The cytotoxicity of AMP on cancer cells (a~d) and normal cells (e~f). a,b. B16BL6, c. A549, d. HT1080, e. RAW264.7, f. HUVEC cell lines

Table 1. Cytotoxicity of AMP in various cell lines.

a.-1. Cytotoxic effect of AMP on B16BL6 cells

Concentration(ug/ml)	62.5	125	250	500	1000
			% of control		
AMP	80.8±3.7[a)]	81.9±4.3	77.5±2.4	67.2±6.8	65.2±3.1

a-2. Cytotoxic effect of AMP on B16BL6 cells

Conc (mg/ml)	1.25	2.5	5	10	20	40
				% of control		
AMP	73.7±8.2	70.1±7.7	66.3±8.3	58.4±7.9	51.6±7.4	0.1±0.2

b. Cytotoxic effect of AMP on A549 cells

Concentration(ug/ml)	125	250	500	1000	2000
			% of control		
AMP	85.1±2.5	85.9±2.3	80.5±1.1	74.0±1.0	62.8±0.8

c. Cytotoxic effect of AMP on HT1080 cells

Concentration(ug/ml)	125	250	500	1000	2000
			% of control		
AMP	90.9±3.7	83.4±4.2	84.7±4.1	86.5±3.3	84.1±3.2

d. Cytotoxic effect of AMP on RAW264.7

Conc (mg/ml)	1	2	5	10	20	40
				% of control		
AMP	87.1±9.4	68.4±9.4	49.0±8.8	40.2±6.5	27.3±6.8	0.1±0.1

e. Cytotoxic effect of AMP on HUVEC

Conc(ug/ml)	62.5	125	250	500	1000
			% of control		
AMP	109.6±10.3	94.8±16.4	87.3±15.6	67.6±10.8	66.3±6.5

a) Mean±S.D

2. Inhibitory effect of AMP water extract on angiogenesis

Angiogenesis is an essential stage for the growth and metastasis of tumor. To investigate antiangiogenic effect of AMP, we performed CAM assay. AMP was treated with 0.5 and 1mg/egg, in which concentration there is nearly no cytotoxicity (Fig. 1. f). The blood vessels below the thermanox of CAM in control group have formed well. On the other hand, AMP treated groups disrupted blood vessel formation of CAM with 50% and 40% inhibition at 1mg/ml and 0.5mg/ml respectively.

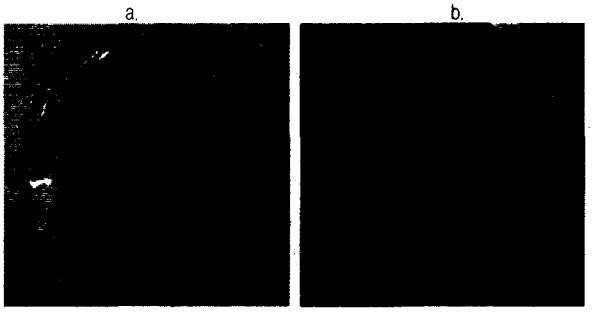

Fig. 2. Phothgraphy of control and AMP(1mg/egg) on embryonic angiogenesis in CAM 2 days after sample topping. a. control group, b. AMP (1mg/egg) treated group

This result can't absolutely suggest antiangiogenic effect, however, it indicates that AMP has the possibility of suppressive effect for the angiogenesis as well as neovascularization. It is, therefore, still necessary to study more not only in migration, tube formation but also molecular biological studies.

Table 2. Antiangiogenic activity of AMP in a CAM assay

Sample	Dose(mg/egg)	No. of CAM (avascular/total)
Control	PBS(10ul)	0/7
AMP	1	4/8
AMP	0.5	2/5

3. Effect of AMP water extract on tumor cell adhesion

During the metastatic cascades, tumor cells encounter host cells or extracellular matrix and basement membrane components[24]. As a result of adhesive interaction, this encounter may lead to be able to enhance the survival, arrest and invasiveness of tumor cells[25-26]. To investigate the suppressive effect of AMP on the tumor cell attachment, tumor cell adhesion assay was used. Figure 3 is showing that AMP restrict the adhesion between B16BL6 and gelatin which is one of the ECM.

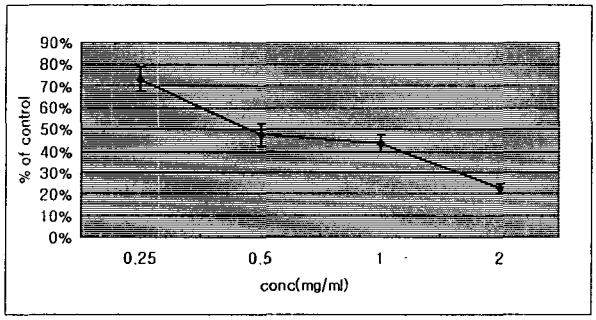

Fig. 3. Antiadhesion effect of AMP on B16BL6 cells

4. Effect of AMP water extract on TCIPA

The tumor cells which have escaped from primary cites will circulate the blood vessel of all over the body. During the circulation, the tumor cells will go through the attack by host cells like macrophage, natural killer cell (NK cell), and the other immunocyte as well as the breakdown of cell membrane by high rapid blood flow. In order to survive from this barrier, it is essential for tumor cell to trick immune system by various means and tumor cells which successfully escaped from this circulation, must adhere endothelial cell of the target organ. Platelets are known to play an important role in the regulation of theses steps[27-29]. The surface antigen of tumor cell may be hidden from the immune system by being enveloped with platelet and tumor cell can easily attach to the endothelial cell of the target organ.

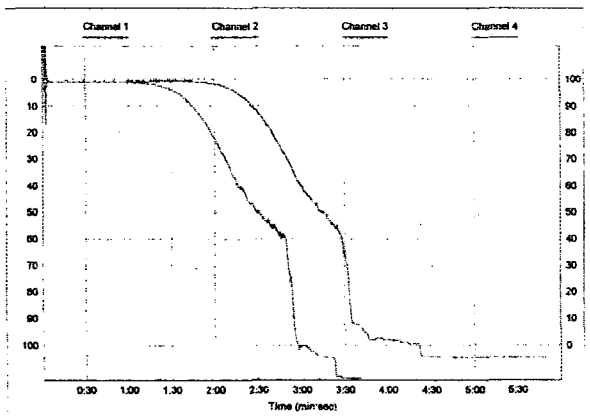

Fig. 4. Surpressive effect of AMP on platelet aggregation with B16BL6 cells. Channel 1 : platelet of control. Channel 2 : 1mg/ml(final concentration) of AMP treated platelet

Thus, the activity of AMP on the aggregation of platelet was examined to find out whether AMP inhibits tumor induced platelet aggregation. Figure 4 shows that the AMP treated platelet began to aggregate later than the control. From this result, AMP can be inferred to inhibit the interaction between the tumor cell and platelet to a slight degree. Consequently, tumor cells are increased the chance of attack by immune system as well as decreased the opportunity of attachment to endothelial cells via the platelet.

5. Suppressive effect of AMP water extract on lung metastasis in mice treated with B16BL6 melanoma cells

To investigate antimetastatic effect of AMP, in vivo experimental lung metastasis of B16BL6 cell lines was performed. Figure 5 shows that i. v. administration of AMP (10 mg/mouse), 3 times (on -2, -1, 0 day) before tumor inoculation and 9 times (on +1, +2, +3, +4, +5, +6, +8, +10, +12 day) after tumor inoculation (on 0 day), significantly inhibited lung metastasis produced by B16BL6 melanoma cells. This result could be explained from various points of view. AMP may show antimetastatic effect by modulating MMP, u-PA, TIMP and so on. AMP could possibly do indirectly through immunomodulatory activity.

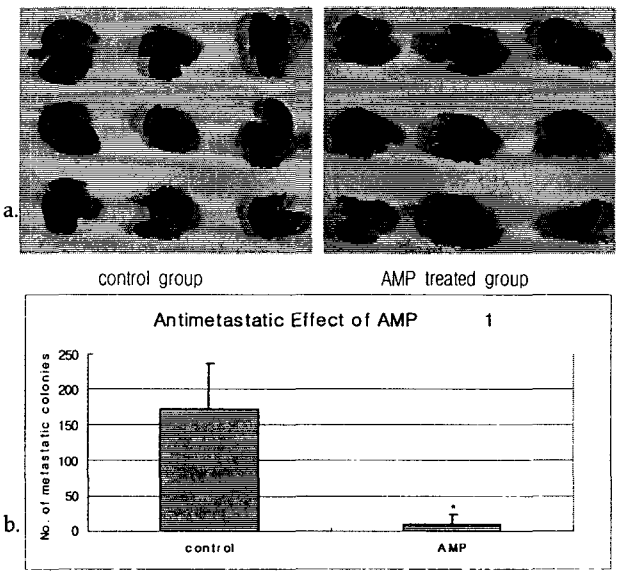

Fig. 5. Antimetastatic effect of AMP(10mg/mouse) on lung colonies in C57BL/6J injected i.v. with B16BL6 cells. a. Photography of lung colonies in C57BL/6J i.v injected. with B16BL6 cells. b. Number of lung colonies. * : statistical significant. P<0.001

Because AMP was orally administrated, AMP probably does not directly work on cells. Recently, a large variety of models of both artificial and spontaneous metastases have been

developed in experimental animals. These models have enabled the characterization of metastatic cancer cells and have helped in understanding the metastatic process[30]. Due to technological developments, we are also able to examine real-time tumor cell trafficking[7]. Studies of experimental metastasis of human tumors, however, have so far been rather limited. We still need more studies on whether the effect of AMP is equally applicable in human body.

6. Effect of AMP on cytokine release in serum

To examine the increasing effect of the natural immunity of AMP, AMP was orally treated to Balb/C mice in vivo three times. Next day, we sacrificed mice to take blood and centrifuged it at 2500rpm for 10 minutes. The supernatant was directly used for cytokine assay. OD value of IL-12 in AMP treated group was higher than control group. IL-12 is a principal mediator of the early innate immune response and is a key inducer of cell-mediated immunity, the adaptive immune response. IL-12 was originally identified as an activator of NK cell, but its most important action is to stimulate INF-r production by T cells as well as NK cells. The principal sources of IL-12 are activated mononuclear phagocytes and dendrite cells. Therefore, AMP can be inferred to activate macrophage to secrete IL-12 and this IL-12 will activate NK cells and T-cell.

Table 3. Effect of AMP treatment on cytokine release

Group	Dose(mg/mouse)	Cytokines(pg/ml)
		IL-12
Control	0	698
AMP	2	706
	10	1135
	50	1160

7. Effect of AMP on extension of Lifespan Time in S-180 injection mice

To investigate whether AMP treatment could prolong the life-span of ICR mice, we used the S-180 injected mice model. The mean span time of AMP treated mice was about four days longer than control group. Although insignificant, the results suggest that AMP has antitumor activity against ICR mice bearing S-180 cells

Table 4. Effect of AMP on extension of Mean Span Time in S-180 injection

Group	Dose (㎎/20g)	Mean Span time(day)	T/C value(%)
Control	0	16.13	100
AMP	2	16.88	104
	10	18.43	114
	50	20.57	127

* T/C value : mean survival time of treated mice/mean survival time of controls

DISCUSSION

Metastasis is one of the greatest characteristics of malignant tumors. In the early stage of blood-borne metastasis, metastasis occurs through a complex cascades including dissociation of cancerous cells from the primary site, intravasation, adhesion to the vascular endothelium of the target organ, extravasation, and growth at the colonization site [31-32]. During this process, a very small percentage (<0.01%) of metastatic cells survive, because host resistance mechanisms for limiting the dissemination and rapid progressive outgrowth of tumor cells may come to prevent metastasis[23]. Therefore, development of metastasis may be the end result of a sequential process including the ability of metastatic cells to circumvent the immune surveillance of the host.

According to diagnostic theory of oriental medicine, Qi-Deficiency and Blood stasis(QDBS) syndrome often was observed in neoplatic patients[33]. QDBS syndrome is including symptoms of enervation, weakness, pallid face, thready pulse, interruption in blood circulation, and so on. Generally speaking, the physiologic function of immune system is a defense against infectious foreign substances. From this perspective, the concept of ZhengQi in oriental medicine is thought to be similar to the Immune system. This syndrome could be cured by supplementing Qi and recovering blood condition. This treatment probably will increase immune system. AMP consists of Qi supplementing herbs such as Ginseng Radix and Atractylodis Macrocephalae Rhizoma, and also Blood-supplementing herbs including Polygoni Multiflori Radix and Lycii Fructus. Also, it contains Ganoderma and Agaricus which were known as antitumor fungi. Especially Ganoderma has Qi and blood supplementing activity. We expected this prescription, AMP to have an antitumor effect through immunomodulating mechanism. In fact, it was reported that antitumor effect of Panax ginseng, Atroctylodes macrocephala and Gonoderma lucidum was mediated by immunomodulating mechanism[13,34-37]. Therefore, AMP composed of these herbs, can be expected to show antitumor activity potently. Although AMP significantly inhibited lung metastasis produced by i.v. injection of B16BL6 melanoma cells, it scarcely inhibited the invasion and migration of B16BL6 melanoma and HT1080 fibrosarcoma cells in vitro (data not shown) and the other in vitro experiments. This suggests that AMP does not act directly and may have the other mechanism. The inhibitory property of platelet aggregation may play a supplementary role. Most of all, the immune system must be a key role in this mechanism, especially by IL-12 mediated immune response.

In this study, we can observe that AMP has a wide antitumor spectrum including an antiangiogenic effect, antimetastatic effect, and suppressive effect of platelet aggregation etc. These efficacies were already reported with constituent herb[14,38-40]. However, most of these studies were not the experiments by oral administration of boiled water extract. In addition, there would be a synergistic effect when administered in the form of composition formula. Even though the individual effect on each step of metastasis is insignificant, taken together these results, we could consider that AMP is a good antimetastatic agent on the whole because AMP may act synthetically in human body.

Recently, complementary/alternative medicine is immensely popular[41]. The study for the prescription like AMP, therefore, will be required in order to accompany this mainstream. Especially, in oriental medicine prevention above everything else was put on importance. Actually, there was a report by epidemiological examination that Panax ginseng may reduce the risk of several types of cancer[42-43] besides therapeutic activities. In other words, it is a kind of preventive medicine through increasing the innate immunity directly and secondhand adaptive immunity. AMP is also expected to diminish the risk of metastasis by modulating immunology and inhibiting angiogenesis.

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