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ARTICLE

The *Monascus* Metabolite Monacolin K Reduces Tumor Progression and Metastasis of Lewis Lung Carcinoma Cells

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Monascus-fermented red mold rice extract (RMRE) offers valuable therapeutic benefits and has been extensively used for centuries in East Asia. *Monascus* secondary polyketide metabolites, including monacolin K (MK) and ankaflavin (AK), have been reported to have antitumor-initiating effects on cancer progression. This paper reports that the oral administration of RMRE dramatically inhibited the metastatic ability of murine Lewis lung carcinoma (LLC) cells in syngeneic C57BL/6 mice caused by the decline of serum vascular endothelial growth factor (VEGF) levels compared with untreated metastatic groups. MK is a key antimetastatic and antiangiogenesis compound in RMRE, as shown by down-regulation of VEGF-stimulated invasive activity in LLC cells by Matrigel-coating transwell and tube-forming assays and reverse transcription−Polymerase Chain Reaction (RT-PCR). Therefore, application of RMRE may serve as a nontoxic natural chemopreventive or antineoplastic agent in the development of cancer adjuvant chemotherapy.

KEYWORDS: Monacolin K; metastasis; angiogenesis; vascular endothelial growth factor (VEGF)

INTRODUCTION

Monascus-fermented red mold rice has been recommended as a dietary supplement for reducing the cholesterol and lipoprotein levels in human blood because it contains monacolin K [MK; an analogue of mevinolin from *Aspergillus terreus* (*1*)]. MK acts by competitively inhibiting 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which is the rate-limiting enzyme of the mevalonate pathway of cholesterol synthesis (*2*). There are a number of scientific reports on the medical or functional food effects which have shown that *Monascus*-fermented red mold rice extracts (RMRE) containing MK have beneficial treatment effects in inflammation, bone fractures, glucose intolerance, Alzheimer's disease, and anticancer progression (*3*,*4*). In addition to MK, *Monascus* spp. produce many types of polyketide secondary metabolites, which have many functional properties, including the orange pigment, monascorubrin, which has been reported to affect suppression of tumor promotion induced by 12-*O*-tetradecanoylphorbol 13-acetate followed by initiation with 7,12-dimethylbenz[*a*]anthracene in mice (*5*). The yellow pigments, ankaflavin (AK) and monascin, have been reported to have cytotoxic/cytostatic activities (*6*, *7*).

Our previous proteomic analysis of the altered protein expression profiles in Caco-2 colorectal adenocarcinoma cells treated with RMRE revealed that the IC_{50} value of the RMRE-treated group was lower than the corresponding value in the MK-treated group (*8*). This indicated that some other antiproliferative action independent of MK may occur in cancer cells during RMRE treatment. The present study, in which we have used the murine Lewis lung carcinoma (LLC)-bearing model to elucidate the properties of secondary metabolites from RMRE, suggests that the combined effects could possibly be synergistic. LLC cells subcutaneously injected into syngeneic C57BL/6 mice provide a quantitative model for investigating the primary inoculated tumor and metastases to the lung of the host, which permits discrimination of the biological effects on local tumor and metastatic disease parameters (*9*). In addition to the in vivo assessment of tumor growth, tumor cell proliferation, and lung metastasis, we analyzed the functional significance of the respective single compound and the interaction of different *Monascus* metabolites in vitro. Our studies also showed that the yellow polyketide, AK, synergizes antiproliferative and apoptotic activities combined with MK. To that end, LLC tumor metastasis associated with vascular endothelial growth factor (VEGF) has been an important component of the process by promoting tumor angiogenesis. Reduction of VEGF expression in LLC cells modulated by MK demonstrated anticancer activities associated with retarding tumor growth and metastatic spread.

The objective of this work was to use an LLC tumor-bearing animal model to identify the potent cytotoxic/cytostatic activities from *Monascus* metabolites. Our resulting data revealed the use of RMRE in augmenting anticancer effects on LLC cells in vitro and in vivo. We suggest that, with the exception of the yellow polyketides and MK, the other active components of *Monascus* play a significant functional role in antitumorigenic activity. It is hoped that this synergism may ultimately result in efficacious regimens for cancer adjuvant therapy after further investigation and clinical trials.

MATERIALS AND METHODS

Chemicals and Reagents. The following were purchased from Sigma Chemical Co. (St. Louis, MO): 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), crystal violet, MK, NaCl, $NaHCO_3$, KCl,

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propidium iodide (PI), sodium dodecyl sulfate (SDS), Triton-X 100, trypsin, and trypan blue. Matrigel was purchased from BD Biosciences (Auckland, New Zealand). Fetal bovine serum (FBS) was purchased from Life Technologies (Auckland, New Zealand). LC-grade acetonitrile was purchased from Merck Co. (Darmstadat, Germany). Ethanol (95%) was purchased from Taiwan Tobacco and Liquor Corp. (Taipei, Taiwan). Dulbecco's modified Eagle's medium (DMEM), penicillin, and streptomycin were purchased from HyClone Laboratories (Logan, UT). All other chemicals were of the highest purity commercially available.

Cell Culture. Murine LLC and SVEC 4−10 endothelial cells were obtained from the Bioresource Collection and Research Center (BCRC) in Taiwan and maintained in DMEM media supplemented with 10% FBS, 1% antibiotic/antimitotic solution, and 0.2% $NaHCO_3$. Cells were cultured in 75 cm^2 flasks and incubated in 5% CO_2/95% humidified air at 37 °C.

Preparation of RMRE and Determination of MK and AK Concentrations. *Monascus purpureus* NTU 568, a mutant with high MK production by solid state fermentation, was used as described previously (*10*). The desired compound, AK, was obtained as described with some modifications (*11*). The effective compound was identified by nuclear magnetic resonance (NMR, Varian Gemini, 200 MHz, FT-NMR; Varian Inc., Palo Alto, CA) and electrospray ionization−mass spectrometry (ESI-MS, Finnigan MAT LCQ; Thermo Electron Co., Waltham, MA) analyses. The concentrations of MK and AK in RMRE were analyzed by high-performance liquid chromatography (HPLC, model L-6200; Hitachi Co., Tokyo, Japan), as described previously (*12*). In this study, the HPLC analyses of MK and AK in RMRE were 10 and 1%, respectively. All extracts were dissolved in DMSO, and the concentration was kept below 0.1% (v/v) in the experimental design.

LLC-Bearing Animal Model. Male 6-week-old C57BL/6 mice were obtained from the Laboratory Animal Center of National Taiwan University College of Medicine. They were given free access to normal diet and water and housed in plastic cages with sterilized wood chip bedding under constant environmental conditions with a 12 h light/dark cycle at a temperature of 23 °C and a relative humidity of 50%. To ensure that toxicity would not be a factor, we medicated a preliminary dosage tolerance survey for the proposed 100 and 200 mg/kg dosages of RMRE using three mice for each dose. The animals were treated by oral gavage with the indicated dose for 28 days. No deaths or weight loss occurred in any of the groups. LLC cells (1×10^6) were suspended in 100 μL of phosphate-buffered saline (PBS) and injected subcutaneously into the right hind limb. Additionally, mice were randomly divided into five and three groups in the first and second animal experiments, respectively. In the first experiment, mice were implanted with LLC cells and then administered MK and an equal concentration of MK in RMRE by oral gavage to determine the in vivo efficacy of the *Monascus* secondary metabolites. Five groups of experimental mice ($n = 6$) were administered distilled water (as vehicle control), 10 mg of MK, 20 mg of MK, 100 mg of RMRE (containing 10 mg of MK), or 200 mg of RMRE (containing 20 mg of MK) per kilogram of body weight orally each day for a period of 4 weeks. In the second experiment, animals were subjected to different treatment regimens following cell injection and sacrificed to measure the incidence of lung metastasis once the tumor volume had reached approximately 5000 mm^3 to eliminate the possibility of an overladen volume of the primary tumor leading to the spread of LLC cells to distant sites.

Primary tumor volumes were estimated once weekly after tumor inoculation by caliper measurements of the short (a) and long (b) axes, using the following equation: tumor volume = $\pi/6 \times a^2 \times b$. Animals were sacrificed by carbon dioxide inhalation at the indicated time, and serum, the primary tumor, and the lungs were collected and used in the following experiments. These experiments were approved by the National Science Council Committee, as well as the Animal Center Committee of the College of Medicine of National Taiwan University in Taiwan.

Lung Histomorphometric Examination. The dissected lung was fixed with 10% neutral phosphate-buffered formalin (Sigma Chemical Co.). The embedded tissue was serially sectioned into 5 μm sections every 200 μm. Paraffin sections were deparaffinized in xylene, rehydrated with graded ethanol, and stained with hematoxylin and eosin (H&E). The H&E staining slides were photographed under a microscope (ECLIPSE TS100; Nikon Co., Tokyo, Japan) and analyzed using National Institutes of Health (NIH) ImageJ software (http://rsb.info.nih.gov/ij/).

Proliferation Inhibition Assay. The antiproliferative effects of MK, AK, and MK + AK against LLC cells were measured using the MTT reduction assay, as described previously (*8*). Cells (5×10^4 cells/well) were plated on 24-well plates in DMEM with 10% FBS. After a 24 h incubation with 10% FBS, the cells were serum-starved for 24 h and then treated with various concentrations of MK, AK, and MK + AK. The results were then obtained spectrophotometrically using a microplate reader (Thermo Lab Systems Opsys MR, Chantilly, VA).

Detection of Cell Viability by Flow Cytometry. To determine the cell cycle phase distribution, LLC cells were seeded in 6 cm culture dishes in DMEM with 10% FBS. After a 24 h incubation, the cells were serum-starved for 24 h and treated with various concentrations of MK, AK, and MK + AK for the indicated times. After 48 h, the cells were resuspended and washed twice with cold PBS and then incubated in PI staining solution (20 μg/mL) for 15 min at room temperature. The stained cells were analyzed by flow cytometry (FACScalibur, Becton Dickinson, NJ) with CellQuest Pro software. Ten thousand cells were recorded per assay.

Quantification of VEGF Protein by ELISA. To quantify mouse serum VEGF proteins, the inferior vena cava of the mouse was punctured and peripheral blood was collected. Simultaneously, tumor and lung tissue lysates containing equivalent amounts of total protein were analyzed for VEGF content by a murine ELISA kit (R&D Systems, Minneapolis, MN).

Matrigel Invasion Assay. Cells (1×10^5) were suspended in serum-free DMEM and seeded in the upper chamber of Matrigel-coated Millicell-HA filters (8 μm pore size; Millipore Co., Bedford, MA). The lower chamber was filled with 10% serum medium containing VEGF (10 ng/mL per chamber) as the chemoattractant. After a 24 h incubation, the filter was gently removed from the chamber, the cells on the upper surface were removed by wiping with a cotton swab, and the cells that had invaded the Matrigel and attached to the lower surface of the filter were fixed, stained for 10 min with 0.5% crystal violet, and counted in five randomly selected microscopic fields (×400) per filter. The experiments were independently performed at least two times.

Reverse Transcription−Polymerase Chain Reaction (RT-PCR). Total RNA was isolated using Trizol (Life Technologies) according to the manufacturer's instructions. One microgram of purified total RNA was used for reverse transcription using SuperScript III (Life Technologies). The reaction mixture was incubated at 42 °C for 1 h, and the reaction was terminated by heating at 70 °C for 10 min. Amplification of the RT product by PCR was performed using Promega Taq DNA Polymerase (Promega Co., Madison, WI). All reactions were performed in a thermal cycler (model 2400; Perkin-Elmer, Norwalk, CT) with the following primers and conditions: VEGF sense, 5′-CTG CTC TCT TGG GTC CAC TGG-3′, and antisense, 5′-CAC CGC CTT GGC TTG TCA CAT-3′, were incubated for 3 min at 94 °C for 1 cycle, 15 s at 94 °C, 30 s at 62 °C, and 1 min at 72 °C for 30 cycles. The expected product sizes for $VEGF_{120}$, $VEGF_{164}$, and $VEGF_{188}$ were 431, 563, and 635 bp, respectively (*13*). The reaction products were separated on 1.5% agarose gel, stained with 1 μg/mL ethidium bromide, and visualized using a UVP GDS-7900 digital imaging system (UVP AutoChemi System, Cambridge, U.K.). All amplifications were conducted within the linear range of the assay, normalized to respective GAPDH levels using primers from Clontech Laboratories, Inc. (Palo Alto, CA), and amplified under optimal conditions. The results were confirmed by conducting at least three replicate experiments.

SVEC 4−10 Capillary Tube Formation Assay. The tube formation assay was performed using BD Matrigel (BD Bioscience). Briefly, solid gels were prepared according to the manufacturer's manual on a 48-well tissue culture plate. Subconfluent SVEC 4−10 cells were harvested with trypsin−EDTA and centrifuged at 300*g* for 5 min. SVEC 4−10 cells in DMEM complete culture medium were used as control. The cell suspension (4×10^5 cells/mL) in the LLC culture-conditional medium containing 0−5 μM MK was seeded at 200 μL per well onto the surface of the solid gel. After 12 h in culture, the tube-like network was stained with Molecular Probes CellTracker Green dye (CMFDA C2925; Invitrogen, Carlsbad, CA) to obtain fluorescent images by adding 1 μM green dye and incubating for 30 min in a 37 °C, 5% CO_2 humidified incubator. Capillary tube formation was observed under an inverted fluorescent light microscope at 40× magnification. Microscopic fields were photographed with a digital camera. The total area of tube structures in each photograph was measured using NIH ImageJ software (http://rsb.info.nih.gov/ij/).

Data Analysis and Statistics. The values are presented as the mean ± SEM. Statistical comparisons between the groups were done using an unpaired, two-tailed Student's *t* test. All comparisons are made relative to controls, and significant differences are indicated as *, $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.001$, respectively.

RESULTS

RMRE Is More Active than MK in Inhibiting Primary Tumor Growth. Our previous study showed that RMRE could reduce Caco-2 cell proliferation more than MK in vitro (*14*). In this study, we demonstrated anticancer activity in RMRE by a compound other than MK. A daily supplement of 200 mg of RMRE per kilogram of body weight caused a significant reduction in tumor volume over the duration of the study (**Figure 1**). The median volume of primary tumor was significantly smaller in the 200 mg of RMRE-treated group (median, 1120 mm^3; range, 609−3021 mm^3) than in the vehicle control group (median, 2638 mm^3; range, 2146−5416 mm^3) by the third week (*, $p < 0.05$). Moreover, the median volumes of the groups treated with 100 and 200 mg of RMRE at the end of the fourth week were 5633 and 3156 mm^3, with ranges of 2679−6528 and 2242−4895 mm^3, respectively. The vehicle control group had a median volume of 6650 mm^3 and a range of 5429−7802 mm^3, which were significantly reduced compared to the groups treated with 100 and 200 mg of RMRE by the fourth week (*, $p < 0.05$, and ***, $p < 0.001$, respectively).

There were no statistically significant differences between the 100 mg of RMRE-treated or 20 mg of MK-treated groups (2277 ± 1183 and 2670 ± 991 mm^3, respectively), although the means were slightly lower than the control group (3544 ± 1172 mm^3) by the third week. Even the 200 mg of RMRE-treated group was more efficacious in inhibiting primary tumor growth, but the 20 mg of MK-treated group, containing the equivalent of MK in 200 mg of RMRE, had no obvious antiproliferative activity compared to the control group at the end of the experiment (6123 ± 652 vs 6819 ± 760 mm^3).

Effects of RMRE on Tumor Weight and Lung Metastases in LLC-Bearing Mice. Beginning on the day of tumor injection, animals were subjected to different treatment regimens during the next 28 days. In the end, they were sacrificed, and the primary tumor weights and the number of metastatic lung nodules were determined. The doses of supplemental RMRE and MK in this study did not affect the amount of food consumed daily by the animals. Also, weight gain before tumor implantation was not significantly different among the groups of animals. Even though the actual body weight plus tumor weight was affected by tumor size, no significant differences in actual body weight gain were noted at necropsy (data not shown).

Table 1 shows the average primary tumor weight of 20 mg in the MK-treated group was decreased relative to the vehicle (distilled water) mice ($p < 0.05$), but not the 10 mg of MK-treated group. The RMRE-treated groups (100 and 200 mg) had decreased tumor weights measured on the 28th day after implantation by 23.6 and 50.8% compared to the LLC-bearing control group (*, $p < 0.05$, and ***, $p < 0.001$, respectively). Similarly, the secondary metastatic tumor nodules, measured at excision on the lung surface, were not visible in animals supplemented with 200 mg of RMRE (mg/kg/day), but only one animal in the 100 mg of RMRE-treated group had three nodules on the lung surface. The other groups treated with 10 and 20 mg of MK had reduced incidences of lung metastasis at five and three of six mice in each group, respectively (**Table 1**).

AK Participates in the Cell Proliferation Process and Induces Apoptosis in LLC Cell Cultures. To rule out the possibility that MK and AK inhibition of cell growth was due to cytotoxic effects,

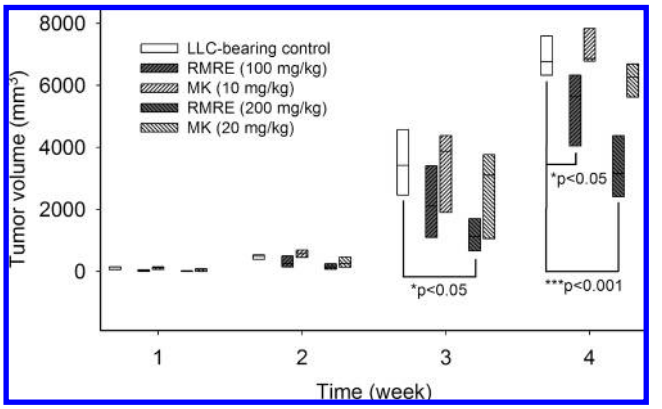

Figure 1. Changes in primary tumor volumes with different treatment regimens. Male C57BL/6 mice were implanted with LLC cells (1×10^6) in the right hind leg and treated with an oral injection of RMRE, MK, or distilled water (vehicle control) at the indicated dose starting from the day of tumor cell inoculation for 28 days. Tumor volume was determined weekly using electronic calipers. The Hi-Low-Close graphs represent the range of tumor volume size in different experimental groups at each indicated time. The bar between each column shows the median volume size of six mice. All comparisons of the mean volume sizes are made relative to vehicle control, and the significance of the difference is indicated as $P < 0.05$ (∗) and $P < 0.001$ (∗∗∗).

Table 1. Effects of RMRE and MK on Tumor Weight and Lung Metastasis in LLC-Bearing Mice

		lung metastasis	
exptl group[a]	tumor wt (g)	incidence[b]	median[c] (range)
LLC-bearing control	8.86 ± 0.99	6	11 (6−18)
RMRE (100 mg/kg)	6.77 ± 1.72*	1	0 (0−3)***
MK (10 mg/kg)	9.30 ± 0.67	5	5.5 (0−11)*
RMRE (200 mg/kg)	4.36 ± 1.27***	0	0 (0−0)***
MK (20 mg/kg)	7.96 ± 0.86*	3	0.5 (0−4)**

[a] LLC-bearing control mice were administered orally once daily 0.1 mL of distilled water for 28 days. Subsequently, the mice of each treated group were administered 0.1 mL of the RMRE (or MK) of their respective concentration orally for 28 days. [b] Number of mice with lung metastases. [c] Median of the number of lung nodules at lung surface, $n = 6$. All of the mice were sacrificed at a particular time; tumor weight values are means ± SE of six mice. *, $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.001$, significantly different from LLC-bearing mice control.

we analyzed the viability and apoptosis of LLC cells treated with MK, AK, and a combination of MK and AK (MK + AK) for 48 h using the MTT assay (**Figure 2A**) and flow cytometric analysis using PI staining (**Figure 2B**). When the cells were treated with MK + AK, there was significant growth inhibition compared with cells treated with MK alone at concentrations of 2.5−10 μg/mL (**Figure 2A**). There was also an obvious increase in the sub-G_1 percentage (70.9 ± 2.4) at 10 μg/mL of MK + AK for 48 h compared with MK or AK treatment alone (**Figure 2B**). However, all three treatments had a similar dose-dependent increase in growth inhibition and the apoptosis assay.

Spontaneous Tumor Metastasis Is Reduced in RMRE- and MK-Treated Mice. RMRE treatment of mice significantly retarded tumor growth, and the mean time to reach the indicated tumor volume was prolonged (**Table 2**). The means of the local tumor weights (g) of the three groups had no statistically significant differences. The incidences of mice with lung metastases at necropsy with tumor volumes of 5000 mm^3 were six and three in the vehicle and MK groups, respectively. The median number of metastatic lung colonies obtained at the lung surface in the MK-treated group was lower than in the vehicle group (**Table 2**).

Histologic Analysis of Lung Tissue with H&E Staining. Differences were observed in lung pathology at the indicated primary tumor size in the second animal experiment. LLC-bearing mice with spontaneous metastases to the lung had been previously demonstrated (*9*). Although the vehicle control group exhibited this representative progression of events, the treatment regimens (RMRE and MK) altered animals significantly different from the vehicle control. In macroscopic features, one of the most obvious differences between the vehicle and MK-treated groups was noted at the gross level (**Figure 3A**,**B**). Moreover, the RMRE-treated group apparently failed to exhibit a granulomatous appearance (**Figure 3C**). For microscopic examination lung tissue was fixed in formalin and embedded in paraffin. H&E staining was then performed on 5 μm thick sections of lung tissue. Numerous metastases were observed on the lung surface of the vehicle and MK-treated groups, whereas no metastases were observed on the surface of the RMRE-treated group. As shown in **Figure 3D**, the percentage of invading cells in the lung as determined by H&E staining was significantly decreased by MK treatment in contrast to the vehicle control (2.4 ± 0.9 versus $18.8 \pm 6.8\%$, respectively). In addition, no lung metastases were observed in the RMRE-treated specimens. The results were calculated using ImageJ online software according to the percentage of metastatic tumor areas of the total section.

Effects of *Monascus* Metabolites on VEGF Expression and Production. Angiogenesis occurs early in tumor progression and

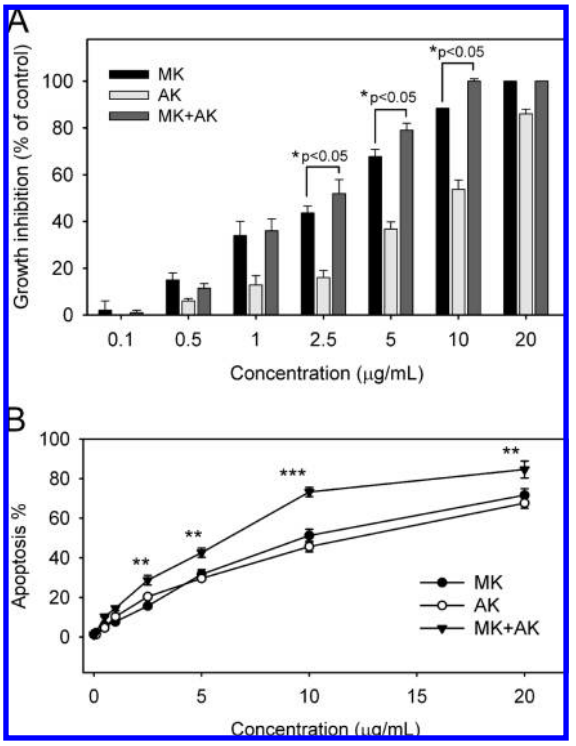

Figure 2. Combination effects of MK and AK on cell growth and apoptosis: dose−effect relationship for LLC cells in growth inhibition and apoptosis assays: (**A**) LLC cells were treated with MK, AK, or MK + AK at the indicated dose. Cell growth inhibition was assessed after 48 h by MTT colorimetric assays. (**B**) LLC cells were treated with MK, AK, or MK + AK at the indicated dose for 48 h. The distribution of cells in the cell cycle was quantified by propidium iodide staining of cells using flow cytometry. Combination of MK plus AK (MK + AK) is at a 10:1 ratio for MK/AK, according to the content percentage in the RMRE. All experiments were performed in triplicate, and the values are expressed in arbitrary units and represent the average and standard deviation of three separate experiments. The synergistic effects of MK + AK significantly different from MK alone are indicated as $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

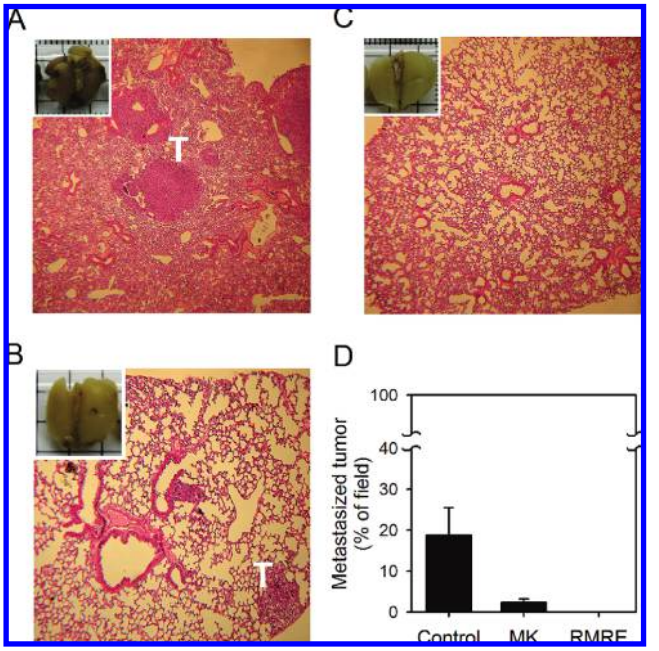

Figure 3. Macroscopic and microscopic features of lung metastases from LLC-bearing mice. Representative macroscopic and microscopic characteristics of the three experimental groups containing distilled water (control, **A**), MK (**B**), and RMRE treatment regimens (**C**) are shown. Animals were treated orally with distilled water (control), MK (20 mg/kg/day), or RMRE (200 mg/kg/day) and scarified to excise the lung for histologic assessment until the primary tumor volume laden was 5000 mm^3. Other than the RMRE-treated group (**C**), distilled water-treated (**A**) and MK-treated (**B**) representative lung images show progression of metastasis, and the arrows point to the metastatic nodules in the lung. Representative hematoxylin and eosin staining sections of the lungs were photographed at 40× magnification; T, tumor. (**D**) Histogram of the measured metastasized tumor areas (μm^2) of the lung section were analyzed with online ImageJ software, and the percentage of metastasized tumor areas was calculated with the following formula: metastasized tumor area (μm^2)/whole filled lung area (μm^2) × 100. The results are shown as the mean percentage of metastasized tumor area of three different fields of sections from six mice per group (two sections per mouse).

Table 2. Frequency of Lung Meatastases in Mice Grafted with 1×10^6 of Lewis Lung Carcinoma Cells

			lung metastasis	
treatment	mean time after tumor laden to 5000 mm^3[a] (days)	tumor wt (g)	incidence[b]	median[c] (range)
distilled water	22.8 ± 3.4	6.62 ± 0.30	6/6	4 (3−6)
20 mg/kg/day MK	24.8 ± 2.4	6.61 ± 0.23	3/6	0.5 (0−2)***[d]
200 mg/kg/day RMRE	36.5 ± 3.3***	6.61 ± 0.21	0/6	0 (0−0)***

[a] All of the mice were sacrificed following tumor volume laden to 5000 mm^3, and the indicated days were means ± SEM of six mice. [b] Number of mice with metastases/total number of mice injected. [c] Median of the number of metastasis per section across the whole lung. [d] $p < 0.001$, significance estimated by the Student's *t* test in comparison to distilled water group.

correlates not only with the onset of tumor development but also with growth and invasion of established tumors (*15*). In this study, we observed the protein levels of VEGF between individual tissues and tumors after LLC cells had been implanted for 28 days. As shown in **Figure 4**, serum VEGF protein levels of RMRE-treated (100 and 200 mg/kg/day) and MK-treated groups (20 mg/kg/day) were 152 ± 8, 84 ± 7, and 126 ± 6 pg/mL, respectively, which were significantly decreased compared with the distilled water-treated control group (185 ± 11 pg/mL). In addition, the tissue VEGF protein levels of the lungs of the RMRE-treated (100 and 200 mg/kg/day) and MK-treated groups (20 mg/kg/day) were 21 ± 2, 16 ± 8, and 22 ± 9 pg/mg, respectively, which were significantly reduced compared with the control group (65 ± 5 pg/mg). However, the VEGF protein concentration in tumors did not show any obvious changes during RMRE or MK treatment. Accordingly, robust VEGF protein levels did not account for the response in the non-LLC cell implanted mice.

Effects of MK on VEGF mRNA Expression and Secretion in LLC Cells in Vitro. In the multiple stages of the metastatic cascade for tumor metastasis, it has been established that angiogenesis is necessary for tumor growth and metastasis. VEGF is one of the most selective and potent angiogenic factors secreted from tumor cells (*16*). Secretory forms of VEGF ($VEGF_{164}$ and $VEGF_{120}$) have been reported to be expressed in mouse tissue and most highly expressed in tumors (*17*). We examined the secretion of VEGF from LLC cells to determine the effect of MK, AK, and MK + AK on invasive activity in LLC cells. Treatment of cells with MK, but not AK, decreased the mRNA expression of VEGF in a dose-dependent manner (**Figure 5A**). MK was more potent than AK in inhibiting VEGF mRNA expression. LLC cells expressed large amounts of $VEGF_{164}$ and $VEGF_{120}$ mRNA. A 24 h treatment of cells with increasing concentrations of MK decreased the expression of $VEGF_{164}$ and $VEGF_{120}$ mRNA (**Figure 5A**). A clear concentration-dependent decrease in MK, but not AK, was observed for a 24 h incubation in a dose-dependent manner. These results indicate that MK was the major candidate in down-regulating the expression of $VEGF_{164}$ and $VEGF_{120}$ mRNA. It may suppress the invasion activity of LLC cell-bearing in mice.

To examine the effects of VEGF-mediated tumor metastasis, we examined the invasion activity of LLC cells in a Matrigel-coated 8 μm pore size Boyden chamber (Millicell-HA, Millipore Co., Bedford, MA). Following subconfluent growth, LLC cells were trypsized and counted, resuspended in serum-free DMEM medium containing MK (0−10 μg/mL), and transferred into the upper compartment of the Boyden chambers (1×10^5 cells/chamber). Recombinant mouse $VEGF_{164}$ (R&D, Minneapolis, MN), as the chemoattracter, was added to the lower compartment containing a 10 ng/mL final concentration in serum-free DMEM medium. **Figure 5B** shows the dose-dependent inhibition with MK of the number of LLC cells that have invaded through the Boyden chambers in response to VEGF chemoattractant. However, MK (> 2.5 μg/mL) significantly inhibited VEGF-stimulated LLC cell invasion (∗, $p < 0.05$). These results indicate VEGF secretion may serve as a candidate marker of metastasis for the malignancy of lung cancer.

Effects of MK on Capillary Tube Formation. We examined the effects of MK from *Monascus* secondary metabolites on endothelial SVEC 4−10 cells. To determine whether LLC secreted VEGF-induced tumor angiogenesis, conditioned medium was harvested at various intervals for measurement of VEGF levels by ELISA. VEGF levels in LLC-conditioned media exhibited a time-dependent increase and plateau at 48 h (**Figure 6A**).

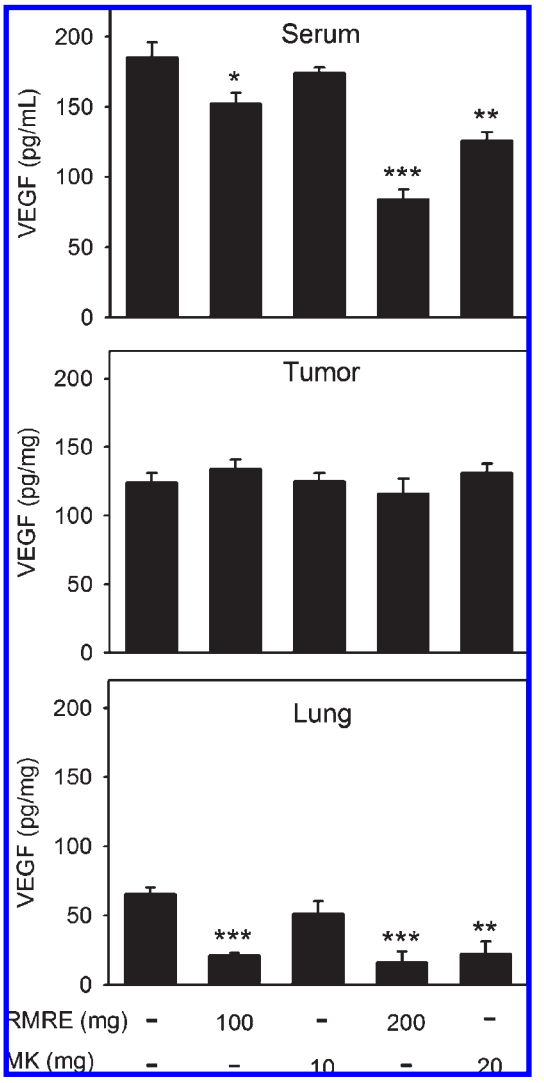

Figure 4. Effects of RMRE or MK on tissue plasma VEGF protein concentration. Mice were treated with an oral injection of RMRE, MK, or distilled water (vehicle control) at the indicated dose starting from the day of tumor cell inoculation for 28 days. Then, VEGF protein levels in serum, tumors, and tissues of lung in LLC-bearing mice were quantified by ELISA. The values represent the mean ± SEM ($n = 6$ per group; ∗, $p < 0.05$, ∗∗, $p < 0.01$, and ∗∗∗, $p < 0.001$, compared to vehicle control group using Student's *t* test).

We then examined the tube-forming activity of these conditioned media pretreated endothelial cells. In the conditioned media-pretreated endothelial cells, tube-forming activity was significantly increased (**Figure 6B**,**C**). On the other hand, LLC culture conditioned medium combined with 1 μM MK pretreatment for 12 h caused a significant suppression in the tube-forming activity (> 30%) of the cells, whereas with MK up to 5 μM, tube forming was essentially inhibited by > 70% (**Figure 6D**−**F**).

In this study, we observed in vitro the secretion and mRNA expression of VEGF, which might be the most important factor for tumor invasion and tumor-induced angiogenesis in LLC-bearing mice. The expression of VEGF was significantly inhibited by MK treatment. These findings suggest that MK may play a critical role in the expression and secretion of VEGF in metastatic cancer cells.

DISCUSSION

In recent years, natural products have received increased attention for the prevention and/or intervention of the early

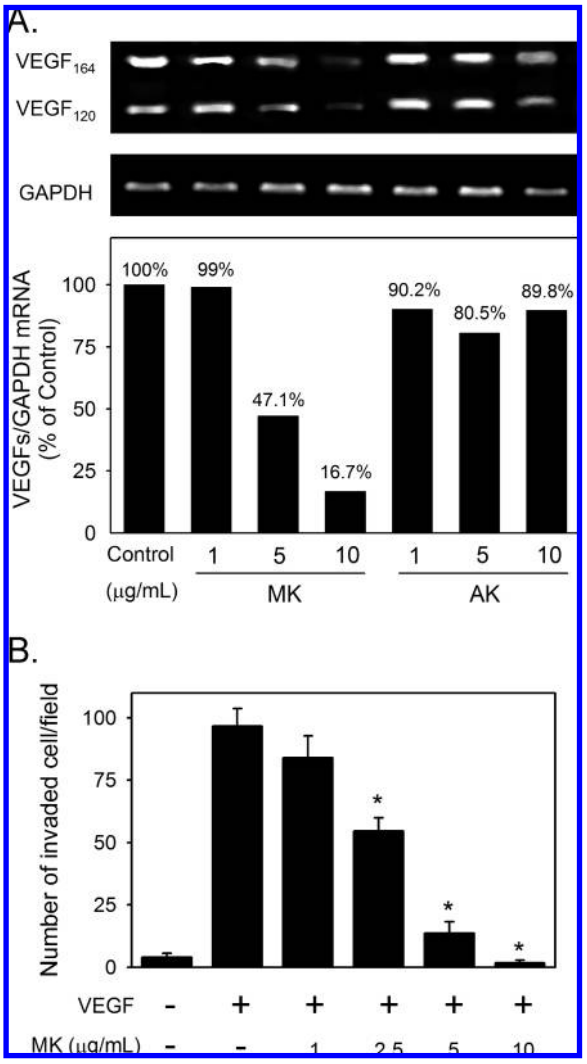

Figure 5. Down-regulation of VEGF decreased LLC cell invasion by MK. (**A**) The expression of VEGF mRNA was measured by RT-PCR using total RNA isolated from LLC cells following different treatments. GAPDH was used as a control. A clear concentration-dependent decrease in MK, but not AK, was observed for an 8 h incubation in a dose-dependent manner. MK was the major candidate in down-regulating the expression of $VEGF_{164}$ and $VEGF_{120}$ mRNA compared with AK. (**B**) The number of invaded cells through Matrigel-coated filters in Boyden chambers was assessed. LLC cells were pretreated or not treated (as negative control) with VEGF (10 ng/mL for 3 h), followed by treatment with MK (0−10 μg/mL) for another 21 h. The invaded cells that attached to the lower surface of the filter coated with Matrigel were counted in five randomly selected microscopic fields per filter. It showed the dose-dependent inhibition with MK of the number of invaded cells that have migrated through the chamber in response to the VEGF chemoattractant. MK (>2.5 μg/mL) significantly inhibited VEGF-stimulated LLC cell invasion (∗, $p < 0.05$).

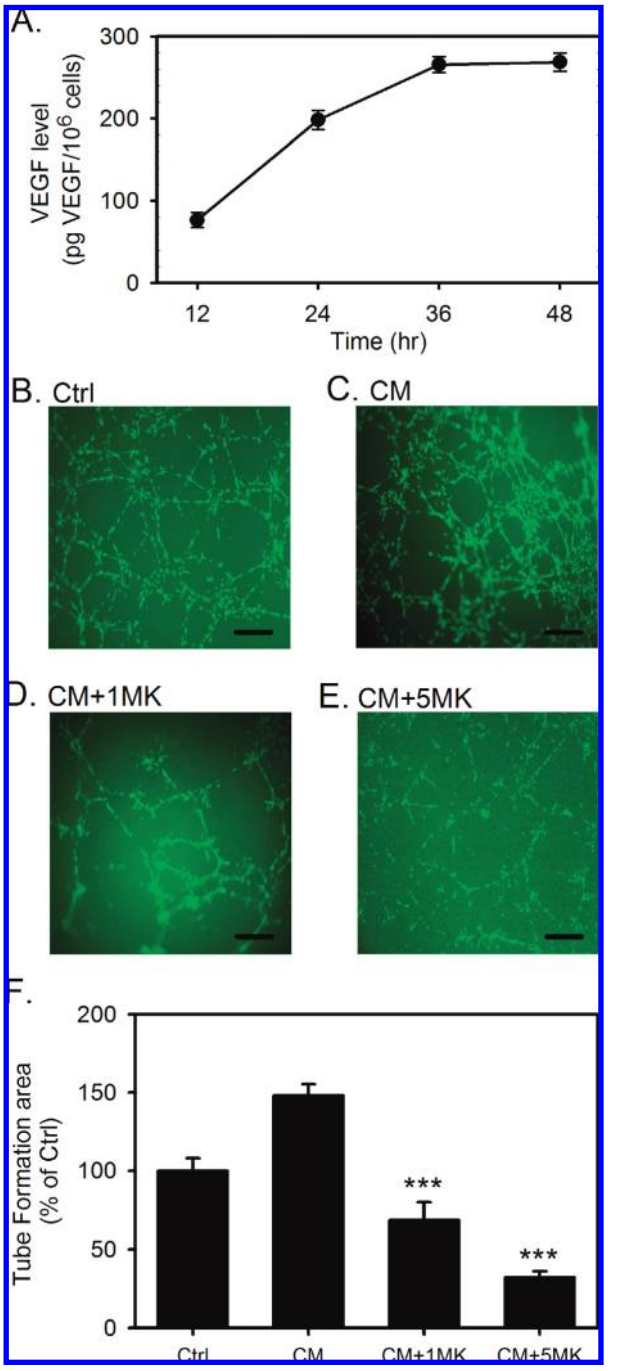

Figure 6. MK inhibits LLC culture-conditioned media-stimulated SVEC 4−10 endothelial tube formation. Tube formation of SVEC 4−10 cells was analyzed by the Matrigel-coating assay. (**A**) LLC cell-conditioned medium was harvested at various intervals for measurement of VEGF levels by ELISA. VEGF levels in LLC-conditioned media exhibited a time-dependent increase and plateau at 48 h. (**B**−**E**) Angiogenic tube formation in SVEC 4−10 cells by different treatments (vehicle control, conditioned media only, and with 1 or 5 μM MK, respectively) for 12 h. Scale bar: 250 μm. (**F**) Total tube area formed by different treatments on SVEC 4−10 cells was analyzed using NIH ImageJ software. All analyses were performed in triplicate. The differences between conditioned media and MK treatment were statistically significant (∗∗∗, $p < 0.001$).

stages of carcinogenesis and neoplastic progression before the occurrence of invasive malignant diseases. Therefore, the secondary metabolites from microbes are regarded as potential chemopreventive agents. A *Monascus*-fermented product is marketed in Asia as a dietetic product, and the preservative effect has also been confirmed by scientific investigation of hypolipidemic agents. Most modern studies on secondary metabolites from *Monascus* have investigated red mold rice and whole broth extract on chronic metabolic diseases effects of hypertension (*18*, *19*), obesity (*20*, *21*), and Alzheimer's disease (*4*). Nevertheless, the chemical characteristics, as well as the biological activities, of many *Monascus*-fermented bioactive agents remain unclear. In this study, we focused on the secondary metabolites, MK and AK, appearing in *Monascus* secondary metabolites, which have been reported to have anticancer activity (*7*, *14*).

Several research groups have focused their efforts on determining the spectrum of the antineoplastic activities of *Monascus* secondary metabolites and have identified different types of tumor cells that exhibit pro-apoptotic or growth suppressive effects (*7*, *22*, *23*). The results of these experiments showed that antiproliferative activity was potentiated when combined with MK plus AK (**Figure 2**). These studies clearly demonstrated cytostatic and antitumor metastasis activity of *Monascus* metabolites in vitro. This is the first study to show that the synergistic effects of MK plus AK enhance activities of apoptosis and antiproliferation on tumor cells.

Herein we describe that pretreatment with a noncytotoxic concentration of RMRE (200 mg/kg/day) dramatically inhibited the metastatic ability of LLC cells in syngeneic C57BL/6 mice. Similarly, the daily treatment of animals with MK (20 mg/kg/day) significantly reduced the number of experimental lung metastases (**Tables 1** and **2**). Thus, it is possible that beyond the chemoprotective effects against cancer, the contents of RMRE may be also found to have a therapeutic role for lung cancer in combination with chemotherapy, but this remains to be assessed in future studies.

Lung cancer is currently the most prevalent malignant tumor worldwide and has the fastest rising incidence and mortality rate of any cancer. Because the majority of cancer deaths results from metastatic complications, understanding cellular alterations contributing to organ-specific metastases is an ongoing cancer research goal. The invasion and metastasis of tumor cells from a primary lesion to distant sites in a target organ are multistep dynamic processes, including angiogenesis, which is regulated to a large extent by myriad angiogenic factors, key among which is VEGF (*24*). Overexpression of VEGF has been shown to provide a significant growth and metastatic advantage to tumorigenic cells implanted into immunodeficient mice (*25*).

The mechanism of micrometastasis of lung cancer is receiving more attention. It is well-known that angiogenesis and invasion are two essential events for metastasis of tumors. VEGF is closely related to angiogenesis, growth, and metastasis of tumors. Secretory forms of VEGF ($VEGF_{164}$ and $VEGF_{120}$) have been reported to be expressed in mouse tissues and most highly expressed in tumors (*17*). Implantation of LLC cells led to a significantly decreased number of metastatic colonies in the *Monascus*-fermented metabolites treatment groups (**Table 2**). Moreover, in vivo evidence from the determination of VEGF protein levels showed that invasion and metastasis were dependent on serum VEGF protein level; in the absence of LLC cell injection, mouse serum showed only low levels of VEGF (data not showed). It is well-documented that VEGF is constitutively overexpressed in tumor cells induced by hypoxia and can increase during tumor growth (*26*). However, we demonstrated that MK exerted antiangiogenic activity through suppressing SVEC 4−10 tube formation and endothelial cell proliferation (**Figure 6**), not only inhibiting VEGF secretion of tumor cells but also retarding cell growth (**Figure 2A**).

In conclusion, we have provided evidence for the first time that RMRE has a potent antitumor activity in vivo on LLC-bearing mice and that a combination of bioactive compounds synergistically increases LLC cell apoptosis and inhibits the proliferative rate in vitro. First, RMRE treatment regimens directly reduced tumor growth and distant metastasis in LLC-bearing mice, and the contents of MK + AK enhanced activities of antiproliferation and apoptosis. Second, the major antimetastatic agent in the present study was MK, but not AK. Third, the secretion of VEGF has been suggested to play a significant role in tumor promotion. MK may play a critical role in the expression and secretion of VEGF in metastatic cancer cells. Finally, this type of antitumor substance from *Monascus* secondary metabolites might create a potential new approach for tumor therapy. Such agents seem to be more promising in clinical practice than the compounds solely targeting tumor progression.

ABBREVIATIONS USED

AK, ankaflavin; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; ESI-MS, electrospray ionization−tandem mass spectrometry; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; H&E, hematoxylin and eosin; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; HPLC, high-performance liquid chromatography; MK, monacolin K; RMRE, *Monascus*-fermented rice extract; NMR, nuclear magnetic resonance spectra; PI, propidium iodide; VEGF, vascular endothelial growth factor.

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