RESEARCH ARTICLE

Oral administration of resveratrol in suppression of pulmonary metastasis of BALB/c mice challenged with CT26 colorectal adenocarcinoma cells

Ya-Ling Weng[1], Hui-Fen Liao[2], Anna Fen-Yau Li[3,4], Ju-Chun Chang[1] and Robin Y.-Y. Chiou[1]

[1] Department of Food Science, National Chiayi University, Chaiyi, Taiwan
[2] Department of Biochemical Science and Technology, National Chiayi University, Chaiyi, Taiwan
[3] Department of Pathology, Taipei General Veterans Hospital, Taipei, Taiwan
[4] School of Medicine, National Yang-Ming University, Taipei, Taiwan

Anti-cancer activities of resveratrol (3,4′,5-trihydroxylstilbene) have attracted extensive research attention. Suppression of pulmonary metastasis of BALB/c mice challenged with CT26 colorectal adenocarcinoma cells achieved by oral administration of resveratrol was assessed in three separate experiments. Each mouse was challenged by tail vein injection with CT26 cells. Prior to challenge, 8-wk-old mice were fed with a basal diet and orally administered with resveratrol (30 mg/kg/2 days) eight or twelve times. After challenge, oral administration of resveratrol was continued until mice were sacrificed on day 20. As integrated from three experiments, 3.7% of the control mice ($n = 27$) and 68.7% of the resveratrol-treated mice ($n = 26$) exhibited free of metastasis. In a second study, 8-wk-old BALB/c mice were orally administered with resveratrol 12 times and challenged with CT26 cells for 100 days. All control mice died but 50% of the resveratrol-treated mice survived. The surviving mice were challenged with CT26 cells by hypodermic injection, fed with a basal diet for an additional 30 days, and sacrificed. Tumor lumps or nodules were not detected at the injection sites or in the lungs. This reveals that intrinsic vaccination-like defense has resulted from administration of resveratrol and challenge of tumor cells.

Received: February 4, 2009
Revised: April 12, 2009
Accepted: April 27, 2009

Keywords:
BALB/c mice / CT26 colorectal adenocarcinoma cells / Oral administration / Pulmonary metastasis / Resveratrol

1 Introduction

Metastasis is defined as spreading of malignant cells from primary cancer sites to form offspring tumors at distant sites. A generalized process of metastasis includes cell detachment from the primary tumor, invasion of the extracellular membrane, entry into vessels and the circulation system, arrest in the capillary bed, adherence to subendothelial basement membranes, entry into organ parenchyma, response to paracrine growth factors, rapid cell proliferation, induction of angiogenesis, and evasion of host immune defenses [1]. For many cancer victims, metastasis is a manifestation of failed treatment and leads to eventual mortality. From the viewpoint of cancer prevention and therapy, it is expected that nutraceuticals or dietary supplements with anti-metastatic potency could be successful. Based on the fact that *in vivo* metastasis involves systemic and multiple interactions, assessment of the efficacy of an anti-metastatic ingredient by animal assays is much more realistic than are *in vitro* experiments. The CT26 colorectal adenocarcinoma cell, an undifferentiated cell line obtained by treatment of the primary cells with *N*-nitroso-*N*-methyl

Correspondence: Professor Robin Y.-Y. Chiou, Department of Food Science, National Chiayi University, 300 University Road, Chiayi, Taiwan
E-mail: rychiou@mail.ncyu.edu.tw
Fax: +8865-2775524

Abbreviations: BUN, blood urea nitrogen; **GOT,** glutamic oxaloacetic acid transaminase; **GPT,** glutamic pyruvic acid transaminase; **LLC,** Lewis lung carcinoma; **RBC,** red blood cell; **WBC,** white blood cell

urethane, is tumorigenic to BALB/c mice. This BALB/c mouse-CT26 tumor model has been used successfully in studying metastasis [2–4].

Many potent bioactivities of resveratrol (3,4′,5-trihydroxylstilbene), a natural phytoalexin detected in grapes, peanuts, and other plants, have been demonstrated. In particular, anti-cancer activities and life span extension properties attributed to resveratrol have attracted extensive research attention [5–17]. Among the activities reported, pulmonary anti-metastasis of colorectal cancer achieved by oral administration of resveratrol has received meager research attention. In toxicological assessments, Sprague–Dawley rats fed resveratrol at 20 mg/kg/day, which is equivalent to the amount consumed by a 70-kg person taking 1.4 g of *trans*-resveratrol/day, for 28 days is not harmful [18]. In another study, CD rats were continuously administered resveratrol at 0, 300, 1000, and 3000 mg/kg/day for 28 days. No kidney injury was observed when rats were challenged with up to 1000 mg/kg/day [19]. This indicates that resveratrol could be orally administered in a broad dose-spectrum of product formulations.

Resveratrol administered intraperitoneally once for 21 consecutive days is effective in prevention of growth and metastasis to lung and tumor-induced neovascularization in Lewis lung carcinoma (LLC)-bearing mice [9]. In that report, anti-metastatic activity of resveratrol being due to inhibition of DNA synthesis in LLC cells and inhibition of LLC-induced neovascularization and angiogenesis was suggested. In addition to direct cytotoxic interaction with tumor cells, the observed potent anti-cancer activities of resveratrol might be accompanied by enhancement of intrinsic defense and/or vaccination-like mechanisms. The immune response of mice has been reported to be enhanced by administration of low dose of resveratrol [20, 21]. Accordingly, the first part experiments of this study were designed mainly to assess pulmonary metastasis of BALB/c mice challenged with CT26 colorectal adenocarcinoma cells as affected by oral administration of resveratrol. Based on extensive preliminary experiments, a dose of 30 mg/kg at 2-day intervals was orally administered eight or twelve times prior to challenge with CT26 cells. In the following part of this study, experiments were done primarily based on the concept of vaccination that anti-metastasis of mice after oral administration of resveratrol might have been potentiated to boost sufficient immune surveillance or suppression [21] against challenge of CT26 cells. It was hypothesized that the surviving mice, after oral administration of resveratrol and challenge with CT26 cells, might have developed a regulatory immune memory and capability to destroy a subsequent challenge with CT26 cells.

More than one century ago [22], immunity to cancer therapy was linked to decrease tumor size when the patient was injected with bacteria or a bacterial metabolite. In 1957, a link between immunity and cancer therapy has been demonstrated [23]. Mice were challenged with a carcinogen to induce tumor formation. Injection of the tumor cells after the tumors were removed by surgery resulted in no further tumor formation. In our study, mice that survived resveratrol treatment and challenge with CT26 tumor cells were subsequently challenged with CT26 cells by hypodermic injection and fed with a basal diet (no resveratrol) for an additional 30 days. Mice were then examined for tumor formation at the injection sites and in the lungs.

2 Materials and methods

2.1 Purity and preparation of resveratrol

To confirm purity, resveratrol obtained from Glory Biotech, Chiayi, Taiwan, isolated from roots of *Polygonum cuspidatum* [9], and an authentic reference source (Sigma-Aldrich, St. Louis, MO, USA) were dissolved in methanol, membrane filtered (0.45 μm), and subjected to HPLC analysis (L-7100 pump, L-7420 UV detector, Hitachi, Tokyo, Japan) with a C18 column (Hypersil ODS column, 250 × 4.6 mm, 5 μm, Thermal Hypersil, Cheshire, UK) [24]. The mobile phase was initially run with 0% solvent A (methanol) and 100% solvent B (deionized water) for 10 min, linearly increased to 30% of A solvent in 10 min, linearly increased to 100% of A solvent in 10 min, and held for an additional 3 min, then decreased to 30% of A solvent in 5 min and 0% of A solvent in 5 min. The flow rate, injection volume, and monitoring wavelength were 1.0 mL/min, 20 μL, and 254 nm, respectively.

To prepare resveratrol solution for oral administration at a dose of 30 mg/kg at 2-day intervals, the required total quantity of resveratrol for each time of oral administration was deposited into a series of brown 1.5-mL Eppendorf tubes and stored at −25°C. Prior to oral administration, an appropriate volume of 30% ethanol (0.1 mL for each mouse) was deposited in a tube, thoroughly mixed, and heated with a thermal module at 65°C for 5 min to enhance solubility. After cooling to ambient temperature (22°C), 0.1 mL of the resveratrol solution was withdrawn using an oral administering gavage and orally administered to each mouse.

2.2 Cultivation of CT26 colorectal carcinoma cells

The CT26 cells were incubated at 37°C under a 5% CO_2 atmosphere in RPMI 1640 (Gibco, Grand Island, NY, USA) as the basal medium. The powdered medium was dissolved in 3 L of deionized water, membrane filtered (0.22 μm), supplemented with 10% fetal bovine serum (Biological Industries, Haemek, Israel) and stored at 4°C until use. Inoculated plates were incubated at 37°C under an atmosphere containing 5% CO_2, and sub-cultured two or three times. For each sub-culture practice, cultivated cells were treated with TEG solution (0.25% trypsin, 0.1% EDTA, and 0.05% glucose in Hanks' balanced salt solution) to prepare cell suspension for subsequent sub-culturing.

2.3 Experiments with BALB/c mice

Murine basal diet (Fusow Industry, Taichung, Taiwan) (formulation shown in Supporting Information) was used to feed mice. Three batches of specific pathogen-free BALB/c ByJNar1 mice for three separate experiments were obtained from National Animal Lab., National Science Council, Taiwan, ROC. For each experiment, four mice with free access to diet and water were raised in each cage in a specific pathogen-free animal facility. The day–night change was set at a 12-h cycle. The temperature and relative humidity were controlled at 20–22°C and 60–65%, respectively. Handling and killing of the mice were in full accordance with the Institutional Animal Care and Use Committee (IACUC) guidelines for the care and management of laboratory animals (NCYU IACUC 94002 and 95001). Diets were provided and drinking water bottles were cleaned and changed weekly. Body weight of each mouse was weighed every 2 days.

Before each experiment, 8-wk-old mice were fed a basal diet for 1 wk for adaptation. A dose of resveratrol (30 mg/kg) was orally administered every 2 days. The dose was equivalent to 15 mg/kg/day and stated in this report without further explication. After oral administration for eight times (16 days) in the first experiment and 12 times (24 days) in the second and third experiments, each mouse, except negative control mice, was challenged with CT26 cells by a tail vein injection of 0.1 mL of CT26 cell suspension. The challenge level was 3×10^5 cells/mouse adjusted by microscopic enumeration of cells after appropriate dilution in sterile PBS solution. As observed in the preliminary experiments, positive control mice usually die on day 20 after challenge of CT26 cells, due to severe pulmonary metastasis. Therefore, the mice were sacrificed on day 20 after challenge of CT26 cells.

2.4 Sacrifice and surgical sampling

Prior to sacrifice, the mice were fasting for 1 day. Each mouse was weighed and sacrificed after anesthesia by subcutaneous injection with 0.1 mL of ketamine/4% xylocaine (2:1, v/v). After chest opening, blood samples were withdrawn from the anterior artery and deposited in two Eppendorf tubes. Blood in one tube was immediately gently mixed with heparin and subjected to blood cell analysis. The other tube was centrifuged at 8000 *g* for 5 min to separate the supernatant serum, which was withdrawn and stored at −20°C for biochemical analyses. Lung, liver, kidney, and spleen were dissected, rinsed with 0.8% saline solution, visually examined, weighed, and immersed in 10% neutral formaldehyde for storage. The lungs were examined and tumor nodules were enumerated.

2.5 Visual and histopathological examination for pulmonary metastasis

Lungs from sacrificed mice were fixed in 10% neutral formaldehyde solution for at least 3 days before visually examining and enumerating tumor nodules. Each pair of lungs was also photographed. The extent of metastasis was classified into four categories according the number of tumor nodules: free-metastasis was 0 nodule/mouse, moderate-metastasis was 1–10 nodules/mouse, extensive-metastasis was 11–100 nodules/mouse, and extreme-metastasis was more than 100 nodules/mouse. For histopathological examination, the dissected lungs were fixed in 10% neutral formaldehyde, dehydrated, and embedded in paraffin. Tissue sections were stained with hematoxylin and eosin, microscopically examined, and photographed. Electronic scanning and area integration of each section was also done. From seven pairs of lungs with low- and middle-metastasis, the correlation between integrated areas of the organ sections and the visually enumerated tumors was further analyzed by linear regression.

2.6 Blood cell and biochemical serum analyses

For blood cell analyses, the freshly collected blood samples (in an ice bath) were immediately subjected to analyses with an automatic blood analyzer (Nihon Kohden MEK 6318J, Nihon Kohden, Tokyo, Japan). This analysis included determinations of white blood cell population (WBC), red blood cell population (RBC), hemoglobin, hematocrit value, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, and platelet population. Biochemical determinations, namely, glutamic oxaloacetic acid transaminase (GOT) and glutamic pyruvic acid transaminase (GPT) activities, blood urea nitrogen (BUN) and creatinine content in each serum sample were analyzed with an automatic biochemical analyzer (Kodak Ektachem DT60 II and DTSC II, Eastman Kodak, New York, NY, USA). Means of values with SDs are reported.

2.7 Survival experiment and second challenge with CT26 cells

Twenty 8-wk-old BALB/c mice were adapted for 1 wk with basal diet and randomly distributed into positive control and resveratrol-treated groups ($n = 10$ in each group). The oral dose of resveratrol was 30 mg/kg at 2-day intervals. After oral administration for 12 times (24-day period), each mouse was challenged with CT26 cells (3×10^5 cells/mouse) by tail vein injection and resveratrol was continuously administered at 2-day intervals for an additional 100 days. Expectedly, one of the control mice died on day 20 after challenge with CT26 cells. During the test period, any mouse that died was subjected to surgery and lungs were visually examined

to enumerate tumor nodules as an indication of extent of pulmonary metastasis. After 100 days, the five surviving mice from resveratrol-treated group were further challenged with CT26 cells (3×10^5 cells/mouse) by hypodermic injection on the right thigh and fed with basal diets (no further resveratrol administration). After an additional 30 days, the mice were sacrificed and lungs were dissected for visual examination and tumor nodule enumeration. The hair on the injection sites was removed by shaving and *in situ* tumors were examined by finger touching and swabbing.

2.8 Statistics

Data were subjected to Student's *t*-test for significance analyses of variance with SigmaStat software (Jandel Scientific, San Rafael, CA, USA). The extent of pulmonary metastasis for each group was measured by integrating bars using Sigmaplot 9.0. The electronically scanned and integrated areas of the tumors detected on the lung sections in correlation with the enumerated numbers of tumor nodules were analyzed by linear regression.

3 Results

3.1 Resveratrol purity and body weight change

The HPLC chromatograms (Fig. 1) of resveratrol used in this experiment and the authentic reference show that their quantitative and qualitative differences were very limited.

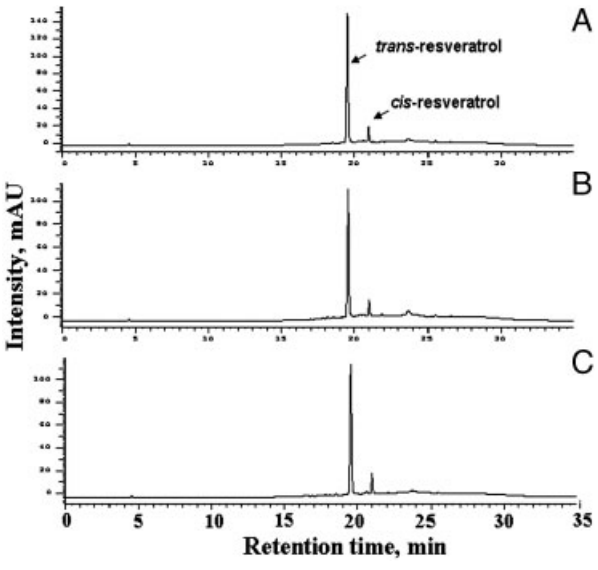

Figure 1. HPLC chromatograms of resveratrol (100 μg/mL) used in this experiment (A); the reference resveratrol (100 μg/mL) (Sigma-Aldrich) and (B) and mixture prepared by equal-volume mixing of (A) and (B) solutions (C).

Both chromatograms contained a predominant peak of *trans*-resveratrol and a minor peak of *cis*-resveratrol [25].

Changes of body weights of the test mice from three separate experiments in which CK^-: negative control (with neither resveratrol treatment nor CT26 challenge); CK^+: positive control (with CT26 challenge but no resveratrol treatment); and R15: treatment with resveratrol at 15 mg/kg/day and CT26 challenge are shown in Fig. 2. In general, body weights of the test mice increased with an increase in feeding time. As shown in Fig. 2I, trends in body weight increase did not deviate among the mice in the negative control (CK^-), positive control (CK^+), and resveratrol-treated groups. Similar trends between the mice of positive control and resveratrol-treated groups were observed in the second and third experiments (Figs. 2II and III).

3.2 Pulmonary metastasis

In the first animal experiment, pulmonary metastasis was detected and is expressed by visual enumeration of the tumor nodules observed on mouse lungs after sacrifice (Figs. 3A1, B1, and C1) and histopathological examination of the lung tissue sections (Figs. 3A2, B2, and C2). Tumor nodules were clearly visible on the lung surface. Intense purple tumor areas were observed in tissue sections stained with hematoxylin and eosin. The extent of pulmonary metastasis detected in test mice after sacrifice, based on lung tumor nodule enumerations, was categorized into four levels, *i.e.* the free (0 nodule/lung), moderate (1–10 nodules/lung), extensive (11–100 nodules/lung), and extreme (>100 nodules). When electronically scanned and integrated tumor cell areas from seven pairs of lungs with moderate and extensive metastasis were analyzed by linear regression of respective corresponding nodule numbers, a linear relationship with 0.89 of co-efficiency was obtained. Accordingly, visual enumeration of the tumor nodules of lungs after sacrifice could be applied as a practical and rational measure of the extent of pulmonary metastasis (all lung photographs are shown in the Supporting Information).

3.3 Suppression of pulmonary metastasis by resveratrol

As shown in Fig. 4A for the first animal experiment, no pulmonary metastasis was examined in the three negative-control mice (CK, with neither resveratrol treatment nor CT26 cell challenge). Among the seven positive control mice challenged with CT26 cells (CK^+), one mouse (10%) exhibited moderate metastasis, four mice (40%) had extensive metastasis, and two mice (20%) showed extreme metastasis. Among the resveratrol-treated mice (R15; 15 mg/kg/day) ($n = 7$), four mice (57.1%) were free of metastasis, two mice (28.6%) had moderate metastasis, one mouse (14.3%) had extensive metastasis, and no mice had

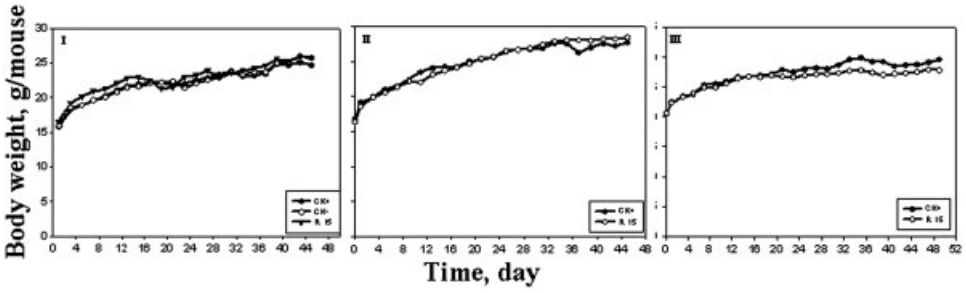

Figure 2. Changes of body weight of BALB/c mice orally administered with resveratrol and challenged with CT26 tumor cells. (I) experiment I; (II) experiment II; (III) experiment III; CK^-: negative control (with neither resveratrol treatment nor CT26 challenge); CK^+: positive control (with CT26 challenge but no resveratrol treatment); and R15: treatment with resveratrol at 15 mg/kg/day and CT26 challenge.

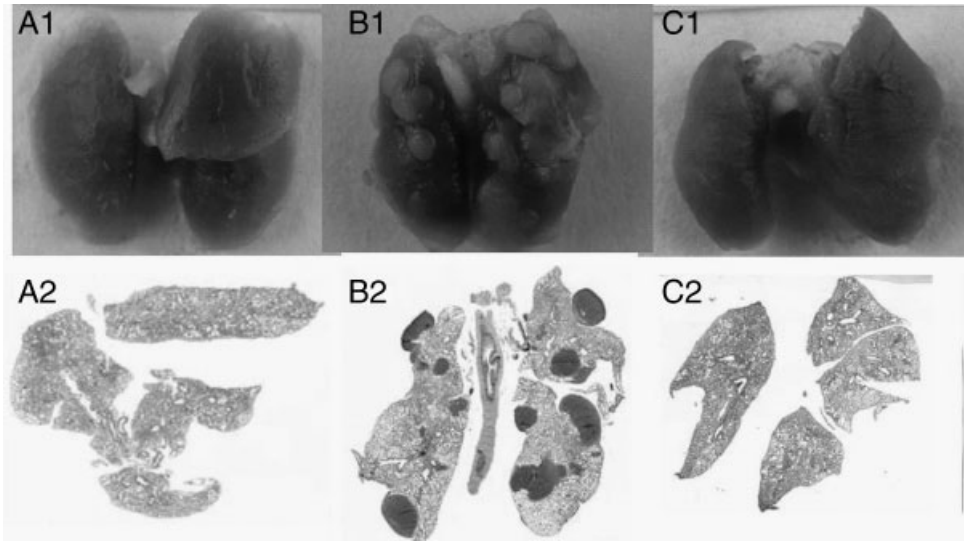

Figure 3. Photographs of lungs and histopathological examinations of the pulmonary metastases of BALB/c mice orally administered with resveratrol and challenged with CT26 tumor cells; (A) negative control (CK^-; with neither resveratrol treatment nor CT26 challenge); (B) positive control (CK^+; with CT26 challenge but no resveratrol treatment); (C) resveratrol treatment (R15; treatment with resveratrol at 15 mg/kg/day).

extreme metastasis. Based on the number of lungs bearing at least one tumor nodule, recognized as positive metastasis, 30% of the positive control mice and 57.1% of the resveratrol-treated mice were free of metastasis.

In the second and third experiments, mice were orally administered with resveratrol at a dose of 30 mg/kg at 2-day intervals 12 times (24 days) prior to challenge with CT26 cells. As shown in Fig. 4B, all positive control mice ($n = 10$, CK^+) showed different extents of metastasis, *i.e.* no mice (0%) were free of metastasis, four mice (40%) had moderate metastasis, three mice (30%) had extensive metastasis, and three mice (30%) had extreme metastasis. For the resveratrol-treated mice ($n = 10$), six mice (60%) were free of metastasis, three mice (30%) had moderate metastasis, and one mouse (10%) had extreme metastasis.

In the third animal experiment (Fig. 4C), one mouse (10%) of the positive control mice (CK^+; $n = 10$) was free of metastasis, four mice (40%) had moderate metastasis, three mice (30%) had extensive metastasis, and two mice (20%) had extreme metastasis. For the resveratrol-treated mice ($n = 9$), eight mice (88.9%) were free of metastasis, no mice (0%) had moderate or extensive metastasis, and one mouse (11.1%) had extreme metastasis. As integrated, an average of 65.4% and a minimum of 57.1% of the resveratrol-treated mice observed in the three separate experiments were free of metastasis. When a comparison was made among the mice with different treatments, 3.7% of the positive control mice ($n = 27$) and 65.4% of the resveratrol-treated mice ($n = 26$) were free of metastasis.

3.4 Survival in suppression of second challenge of CT26 cells

When BALB/c mice were orally administered with resveratrol (30 mg/kg at 2-day intervals) 12 times (over a 24-day period) and challenged with CT26 cells (3×10^5 cells/mouse), and continuously administered with resveratrol for 100 days, one of the positive control mice died on day 20 after CT26 challenge. On days 41 and 100 after challenge, the survival percentages for the control- and resveratrol-treated mice were 80 and 100% and 0 and 50%, respectively (Fig. 5). In the 100-day period after the CT26 challenge, the life spans for the test mice in each group were 54.3 ± 32.2

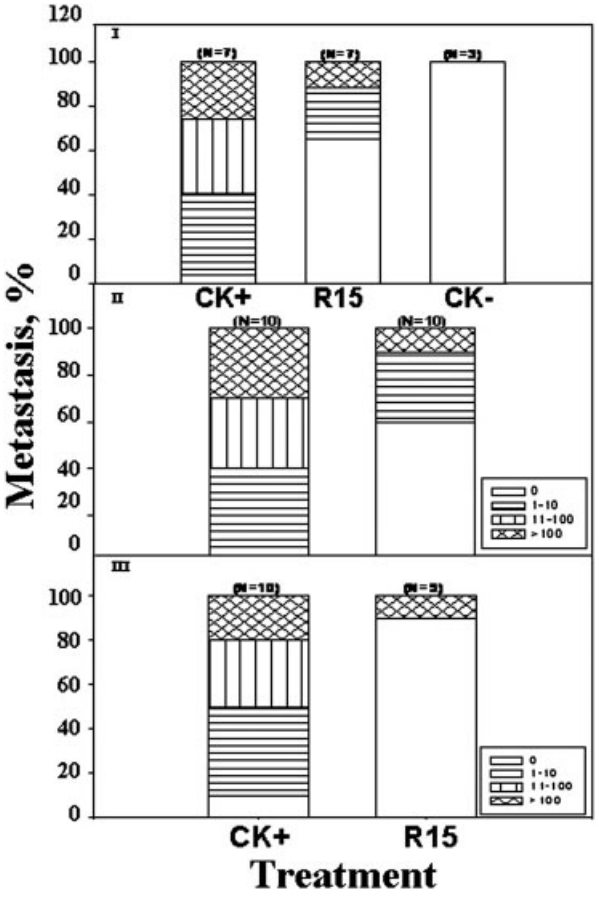

Figure 4. Pulmonary metastasis expressed by lung tumor nodules of BALB/c mice orally administered with resveratrol and challenged with CT26 tumor cells in the first experiment (I), the second animal experiment (II), and the third animal experiment (III); CK^-: negative control (with neither resveratrol treatment nor CT26 challenge); CK^+: positive control (with CT26 challenge but no resveratrol treatment); and R15: treatment with resveratrol at 15 mg/kg/day and CT26 challenge.

and 87.7 ± 23.4 days. When each of the five surviving mice was further challenged with CT26 cells (3×10^5 cells/mouse) by subcutaneous injection of the right thigh and no more resveratrol was orally administered for an additional 30 days, none of the mice died and tumor nodules were not detected at the injection site or in the lungs after sacrifice. Upon further examination of the organs, livers or kidneys were normal. The second challenge of the CT26 cells clearly did not cause further tumor formation.

3.5 Blood analyses

Blood cell analyses (Table 1) showed normal distributions in the number of WBCs ($2.5–15 \times 10^3$ cells/μL), RBCs ($7–12.5 \times 10^6$ cells/μL), hemoglobin contents (10.2–16.6 g/dL), hematocrit values (35–50%), mean corpuscular volumes

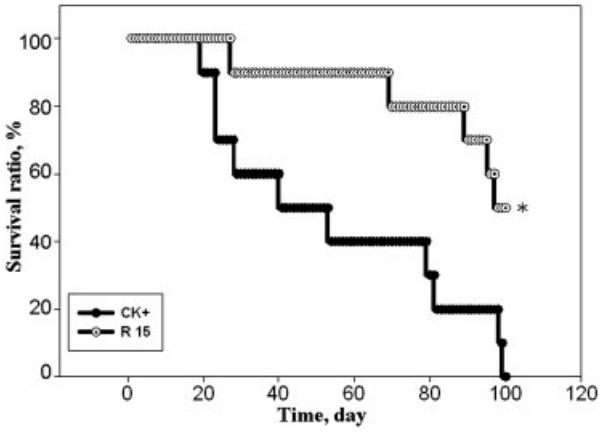

Figure 5. Survival curves of BALB/c mice orally administered with resveratrol and challenged with CT26 tumor cells; CK^+: positive control (with CT26 challenge but no resveratrol treatment); R15: treatment with resveratrol at 15 mg/kg/day and CT26 challenge. *Significantly different ($p<0.05$) from the positive control group as determined by analysis of variance followed by Student's *t*-test.

(14–20 *pg*), mean corpuscular hemoglobin concentrations (25.0–35.5 g/dL), and platelet populations ($160–410 \times 10^3$ cells/μL) for all test mice subjected to challenge with CT26 tumor cells or orally administered with resveratrol and challenge with the tumor cells. All measurements for the test mice were in normal ranges. Limited variations existed among the test groups and among the experiments. The serum GOT and GPT activities, as indicators of liver injury, were in the normal ranges of 59–247 and 28–132 IU/L, respectively (Table 1).

4 Discussion

As accomplished by the three separate experiments, body weights of the test mice increased with an increase in feeding time and trends in body weight increase did not deviate among the mice in negative control (CK; with neither resveratrol treatment nor CT26 challenge), positive control (CK^+; with CT26 challenge but no resveratrol treatment), and resveratrol-treated (R15: treatment with resveratrol at 15 mg/kg/day) groups (Fig. 2). This was in agreement with a study reporting that administering resveratrol at 20 mg/kg/day for 28 days is not harmful [18]. In another study, rats were fed with resveratrol at doses up to 300, 1000, and 3000 mg/kg/day for 28 days [19]. There was no difference in body weight among the female and male rats fed 300 mg/kg/day or male rats fed 1000 mg/kg/day. In the same report, decreases in body weight for the female rats fed with 1000 and 3000 mg/kg/day and male rats fed with 3000 mg/kg/day were observed.

In this study, pulmonary metastasis was detected and is expressed by visual enumeration of the tumor nodules observed on mouse lungs after sacrifice (Figs. 3A1, B1, and

Table 1. Hematological and biochemical determinations of BALB/c mice challenged with CT26 tumor cells (CK^+: positive control with CT26 challenge but no resveratrol treatment) and mice orally administered with resveratrol and challenged with CT26 tumor cells (R15: treatment with resveratrol at 15 mg/kg/day and CT26 challenge)

	Experiment I		Experiment II		Experiment III	
Items	CK^+ ($n=7$)	R15 ($n=9$)	CK^+ ($n=10$)	R15 ($n=10$)	CK^+ ($n=10$)	R15 ($n=9$)
White blood cells, 10^3 cells/μL	2.7±0.4	2.6±0.4	8.1±0.6	3.9±1.0	6.2±2.0	7.4±2.1
Red blood cell, 10^6 cells/μL	8.1±0.5	7.8±0.5	8.1±0.8	6.4±1.1	10.5±0.6	10.1±0.5
Hemoglobin content, g/dL	12.0±0.7	11.5±0.8	7.4±1.2	8.3±0.6	14.1±0.7	13.7±0.7
Hematocrit, %	43.6±2.7	42.1±2.9	34.3±11.3	34.1±6.1	55.1±3.9	52.6±3.5
Mean corpuscular volume, fL	51.0±3.0	49.1±3.3	55.5±2.1	53.1±2.6	52.4±1.7	51.7±2.4
Mean corpusular hemoglobin, pg	14.1±0.8	13.5±0.9	13.3±0.4	13.1±1.4	13.4±0.2	13.4±0.2
Mean corpusular hemoglobin concentration, g/dL	25.3±1.5	24.4±1.6	24.1±0.8	24.7±2.8	25.6±1.1	26.1±1.6
Platelet, 10^3 cells/μL	279.8±24.4	267.0±24.4	155.8±33.4	273.9±40.0	192.2±32.0	151.5±25.5
GOT, IU/L	233.9±10.7	147.3±29.2	103.6±53.7	131.1±83.6	115.2±48.6	115.0±28.5
GPT, IU/L	52.3±16.2	61.2±26.1	38.1±13.0	38.1±12.9	34.0±14.6	32.8±10.2
BUN, mg/mL	13.0±3.9	13.1±4.4	25.8±1.6	25.7±2.2	23.0±3.0	25.7±2.2
Creatinine, mg/dL	0.3±0.0	0.4±0.3	0.6±0.2	0.4±0.1	0.4±0.0	0.4±0.0

Mean of determinations with standard deviation.

C1) and histopathological examination of the lung tissue sections (Figs. 3A2, B2, and C2). Based on the fact that a linear relationship with 0.89 of co-efficiency was obtained by a regression analysis between the electronically scanned and integrated tumor cell areas from seven pairs of lungs with their respective corresponding nodule numbers, visual enumeration of the tumor nodules of lungs after sacrifice was applied as a practical and rational measure of the extent of pulmonary metastasis. As shown in Fig. 4 for the three separate animal experiments, it is noteworthy that oral administration of resveratrol is of efficacy in suppression of pulmonary metastasis. An average of 65.4% and a minimum of 57.1% of the resveratrol-treated mice were free of metastasis. When a comparison was made among the mice with different treatments, 3.7% of the positive control mice ($n=27$) and 65.4% of the resveratrol-treated mice ($n=26$) were free of metastasis. There is no doubt that pulmonary metastasis of CT26 cell was effectively suppressed by oral administration of resveratrol. Even anti-cancer activities of resveratrol have been extensively investigated and reported [5–14]; this study demonstrated a further merit of advancement in the use of resveratrol as a dietary supplement in suppression of pulmonary metastasis of colon cancer.

In addition, it is of further interest to observe when BALB/c mice were orally administered with resveratrol 12 times and challenged with CT26 cells, and continuously administered with resveratrol for 100 days, the survival percentages for the control- and resveratrol-treated mice were 0 and 50%, respectively (Fig. 5). This was in agreement with a similar experiment done with A/J mice in which mice were systemically injected with resveratrol at a dose of 40 mg/kg/day and challenged with neuro-2a cells (1×10^6 cells/mouse) for 28 days [26]. In that report, the survival percentage in the resveratrol-treated group was 70%, achieved by systemic injection of resveratrol. In another report, resveratrol administered intraperitoneally once for 21 consecutive days is effective in prevention of growth and metastasis to lung and tumor-induced neovascularization in LLC-bearing mice [9]. In our study, resveratrol was orally administered. When each of the five surviving mice was further challenged with CT26 cells by subcutaneous injection of the right thigh and no more resveratrol was orally administered for an additional 30 days, none of the mice died and tumor nodules were not detected at the injection site or in the lungs after sacrifice. Upon further examination of the organs, livers or kidneys were normal. The second challenge of the CT26 cells clearly did not cause further tumor formation. The mechanisms involved are unclear after injection with resveratrol; challenge of the CT26 cells may have conferred intrinsic capability in suppressing cancer metastasis. Resistance against a second challenge with the same cancer cells is of merit and worth further investigation.

Based on the reported findings [9, 14] and our results indicating that anti-metastasis was observed in various mouse models after resveratrol administration and resveratrol is regarded as having low cytotoxicity [18, 19], effectiveness of resveratrol in enhancing immunity and other defense against cancer cells is likely. The importance and enhancement of immune surveillance by resveratrol in cancer prevention and/or therapy have been demonstrated [20]. With regard to vaccination, the resveratrol-administered mice that survived the challenge of CT26 cells might have developed an anti-cancer mechanism to destroy the second challenge with CT26 cells. In our study, the percentages (60 and 88.9%) of mice free of metastasis after administering resveratrol 12 times prior to challenging with CT26 cells (experiments 2 and 3) were higher than the percentage in mice subjected to administration of resveratrol eight times (57.1% in experiment 1) (Fig. 4). This reveals that duration

of resveratrol administration is important for mice to potentiate anti-metastatic efficacy after challenge with CT26 cells. Coley [22] in 1893 was the first to link immunity to cancer therapy by observing that tumor size decreased when the patient was injected with infectious bacteria or a bacterial metabolite. Prehn and Main [23] further demonstrated this likelihood. In their report, there is no further tumor formation for the mice that had been induced with carcinogen to bear tumors followed by removal by surgery, after re-injection of the tumor cells. Resveratrol may play a similar estrogenic role as 17 β-estradiol to decrease the IFN-γ and IL-10 ratio and change the Th1/Th2 ratio to favor Th2 to enhance anti-inflammation and immunomodulation [26]. In a recent report [21], intraperitoneal administration of EG7 tumor-bearing C57BL/6 mice with 4 mg/kg resveratrol is effective in suppression of $CD4^+CD25^+$ cell population among $CD4^+$ cells and down-suppression of secretion of TGF-β, an immunosuppressive cytokine. However, as reported by Kimura and Okuda [9], the inhibitory effects of resveratrol on tumor growth and lung metastasis could not be explained by natural killer or cytotoxic T-lymphocyte activation after enumeration of the $CD4^+$, $CD8^+$, and $NK1.1^+$ T cells in the spleens of resveratrol-treated mice. Nevertheless, these observations confirm anti-metastasis activity of resveratrol but the real involved mechanisms remain unclear.

Blood cell and serum analyses for all test mice subjected to challenge with CT26 tumor cells or orally administered with resveratrol and challenge with the tumor cells (Table 1) showed all measurements for the test mice were in normal ranges. Limited variations existed among the test groups and among the experiments. As compared with the data among experiments, differences of mean values of some determinations might be due to blood samples of experiment 1 and samples of experiments 2 and 3 were subjected to analyses by two different laboratories with likely difference in blank calibration. Variations in body weights of the test mice were also limited (Fig. 1). This suggests that administration of resveratrol at a dose of 15 mg/kg/day was harmless to the test BALB/c mice. This is in agreement with a report on rats that were fed resveratrol at a dose of 20 mg/kg/day for 28 days. In that report, RBC, WBC, and platelet numbers and glucose, cholesterol, TG, HDL, LDL, GOT, GPT, BUN, total protein, creatinine, urea, sodium, potassium, chloride, calcium, and inorganic contents were all in normal ranges.

In conclusion, based on three separate animal experiments, oral administration of resveratrol at a dose of 15 mg/kg/day was effective as a chemopreventive treatment for pulmonary metastasis of the challenged CT26 cells. More than 57.1% of the CT26-challenged BALB/c mice treated with resveratrol were free of tumor nodules in their lungs. Of further merit is the observation that resveratrol-treated mice that survived were highly resistant (100%) to tumor colonization by the second challenge of CT26 cells. This indicates that intrinsic defense might have resulted from administering resveratrol and challenge of tumor cells. Vaccination-like immune activation [20, 21] and memory mediation by challenge of tumor cells to real mechanistic investigations of anti-metastasis and anti-tumorigenesis deserve research interests.

The financial support by the National Science Council (NSC 95-2313-B 415-001 and NSC 96-2321-B415-002), Republic of China, valued advice and discussion on manuscript preparation by Dr. Larry R. Beuchat, University of Georgia, USA, and helpful assistance in the laboratory by Tzu-Yuan Lai and Show-Phon Learn are acknowledged.

The authors have declared no conflict of interest.

5 References

[1] Bogenrieder, T., Herlyn, M., Axis of evil: molecular mechanisms of cancer metastasis. *Oncogene* 2003, *22*, 6524–6536.

[2] Wang, M., Chen, P. W., Bronte, V., Rosenberg, S. A. *et al.*, Anti-tumor activity of cytotoxic T lymphocytes elicited with recombinant and synthetic forms of a model tumor-associated antigen. *J. Immunother. Emphasis Tumor Immunol.* 1995, *18*, 139–146.

[3] Liao, H. F., Chen, Y. Y., Liu, J. J., Hsu, M. L. *et al.*, Inhibitory effect of caffeic acid phenethyl ester on angiogenesis, tumor invasion, and metastasis. *J. Agric. Food Chem.* 2003, *51*, 7907–7912.

[4] Chang, K. H., Liao, H. F., Chang, H. H., Chen, Y. Y. *et al.*, Inhibitory effect of tetrandrine on pulmonary metastases in CT26 colorectal adenocarcinoma-bearing BALB/c mice. *Am. J. Clin. Med.* 2004, *32*, 863–872.

[5] Jang, M., Cai, L., Udeani, G. O., Slowing, K. V. *et al.*, Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science* 1997, *275*, 218–220.

[6] Carbo, N., Costelli, P., Baccino, F. M., Lopez-Soriano, F. J. *et al.*, Resveratrol, a natural product present in wine, decreases tumor growth in a rat tumor model. *Biochim. Biophys. Res. Commun.* 1999, *254*, 739–743.

[7] Fremont, L., Biological effects of resveratrol. *Life Sci.* 2000, *66*, 663–673.

[8] Bhat, K. P., Pezzuto, J. M., Cancer chemopreventive activities of resveratrol. *Ann. NY Acad. Sci.* 2001, *957*, 210–229.

[9] Kimura, Y., Okuda, H., Resveratrol isolated from *Polygonum cuspidatum* root prevents tumor growth and metastasis to lung and tumor-induced neovascularization in Lewis lung carcinoma-bearing mice. *J. Nutr.* 2001, *131*, 1844–1849.

[10] Kozuki, Y., Miura, Y., Yagasaki, K., Resveratrol suppresses hepatoma cell invasion independently of its anti-proliferative action. *Cancer Lett.* 2001, *167*, 151–156.

[11] Aggarwal, B. B., Bhardwaj, A., Aggarwal, R. S., Seeram, N. P. *et al.*, Role of resveratrol in prevention and therapy of

cancer: preclinical and clinical studies. *Anticancer Res.* 2004, *24*, 2783–2840.

[12] Aggarwal, B. B., Shishodia, B., *Resveratrol in Health and Diseases*, CRC Press, Boca Raton 2006.

[13] Shakibaei, M., Harikumar, K. B., Aggarwal, B. B., Resveratrol addiction: to die or not to die. *Mol. Nutr. Food Res.* 2009, *53*, 115–128.

[14] Tang, F.-Y., Su, Y.-C., Chen, N.-C., Hsieh, H.-S. *et al.*, Resveratrol inhibits migration and invasion of human breast-caner cells. *Mol. Nutr. Food Res.* 2008, *52*, 683–691.

[15] Howitz, K. T., Bitterman, K. J., Cohen, H. Y., Lamming, D. W. *et al.*, Small molecule activators of sirtuins extend *Saccharomyces cerevisiae* lifespan. *Nature* 2003, *425*, 191–196.

[16] Baur, J. A., Pearson, K. J., Price, N. L., Jamieson, H. A. *et al.*, Resveratrol improves health and survival of mice on a high-calorie diet. *Nature* 2006, *444*, 337–342.

[17] Valenzano, D. R., Terzibasi, E. T., Genade, T., Cattaneo, A. *et al.*, Resveratrol prolongs lifespan and retards the onset of age-related markers in a shortlived vertebrate. *Curr. Biol.* 2006, *16*, 296–300.

[18] Juan, M. E., Vinardell, M. P., Planas, J. M., The daily oral administration of high doses of *trans*-resveratrol to rats for 28 days is not harmful. *J. Nutr.* 2002, *132*, 257–260.

[19] Crowell, J. A., Korytko, P. J., Morrissey, R. L., Booth, T. D. *et al.*, Resveratrol-associated renal toxicity. *Toxicol. Sci.* 2004, *82*, 614–619.

[20] Feng, Y. H., Zhou, W. L., Wu, Q. L., Li, X. Y. *et al.*, Low dose of resveratrol enhanced immune response of mice. *Acta Pharmacol. Sci.* 2002, *23*, 893–897.

[21] Yang, Y., Paik, J. H., Cho, D., Cho, J.-A. *et al.*, Resveratrol induces the suppression of tumor-derived $CD4^+CD25^+$ regulatory T cells. *Int. Immunopharmacol.* 2008, *8*, 542–547.

[22] Coley, W. B., The treatment of malignant tumors by repeated inoculations of erysipelas. *Am. J. Med. Sci.* 1893, *105*, 3–11.

[23] Prehn, R. T., Main, J. M., Immunity to methylcholantrene-induced sarcomas. *J. Nat. Cancer Inst.* 1957, *18*, 769–778.

[24] Chang, J. C., Lai, Y. H., Djoko, B., Wu, P. L. *et al.*, Biosynthesis enhancement and antioxidant and anti-inflammatory activities of peanut (*Arachis hypogaea* L.) arachidin-1, arachidin-3, and isopentadienyl resveratrol. *J. Agric. Food Chem.* 2006, *54*, 10281–10287.

[25] Trela, B. C., Waterhouse, A. L., Resveratrol: isomeric molar absorptivities and stability. *J. Agric. Food Chem.* 1996, *44*, 1253–1257.

[26] Chen, Y., Tseng, S. H., Lai, H. S., Chen, W. J., Resveratrol-induced cellular apoptosis and cycle arrest in neuroblastoma cells and antitumor effects on neuroblastoma in mice. *Surgery* 2004, *136*, 57–66.

[27] Rachon, D., Rimoldi, G., Wuttke, W., In vitro effects of genistein and resveratrol on the production of interferon-gamma (IFNgamma) and interleukin-10 (IL-10) by stimulated murine splenocytes. *Phytomedicine* 2006, *13*, 419–424.

