

Resveratrol Isolated from *Polygonum cuspidatum* Root Prevents Tumor Growth and Metastasis to Lung and Tumor-Induced Neovascularization in Lewis Lung Carcinoma-Bearing Mice

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ABSTRACT Resveratrol is a naturally occurring phytoalexin found in medicinal plants. We found that resveratrol, at doses of 2.5 and 10 mg/kg, significantly reduced the tumor volume (42%), tumor weight (44%) and metastasis to the lung (56%) in mice bearing highly metastatic Lewis lung carcinoma (LLC) tumors, but not at a dose of 0.6 mg/kg. Resveratrol did not affect the number of CD4⁺, CD8⁺ and natural killer (NK)1.1⁺ T cells in the spleen. Therefore, the inhibitory effects of resveratrol on tumor growth and lung metastasis could not be explained by natural killer or cytotoxic T-lymphocyte activation. In addition, resveratrol inhibited DNA synthesis most strongly in LLC cells; its 50% inhibitory concentration (IC₅₀) was 6.8 μmol/L. Resveratrol at 100 μmol/L increased apoptosis to 20.6 ± 1.35% from 12.1 ± 0.36% (*P* < 0.05) in LLC cells, and decreased the S phase population to 22.1 ± 1.03% and 29.2 ± 0.27% from 35.2 ± 1.72% (*P* < 0.05) at concentrations of 50 and 100 μmol/L, respectively. Resveratrol inhibited tumor-induced neovascularization at doses of 2.5 and 10 mg/kg in an in vivo model. Moreover, resveratrol significantly inhibited the formation of capillary-like tube formation from human umbilical vein endothelial cells (HUVEC) at concentrations of 10–100 μmol/L; the degree of the inhibition of capillary-like tube formation by resveratrol was 45.5% at 10 μmol/L, 50.2% at 50 μmol/L and 52.6% at 100 μmol/L. Resveratrol inhibited the binding of vascular endothelial growth factor (VEGF) to HUVEC at concentrations of 10–100 μmol/L, but not at concentrations of 1 and 5 μmol/L. The degree of inhibition of VEGF binding to HUVEC by resveratrol was 16.9% at 10 μmol/L, 53.2% at 50 μmol/L and 47.8% at 100 μmol/L. We suggest that the antitumor and antimetastatic activities of resveratrol might be due to the inhibition of DNA synthesis in LLC cells and the inhibition of LLC-induced neovascularization and tube formation (angiogenesis) of HUVEC by resveratrol. *J. Nutr.* 131: 1844–1849, 2001.

KEY WORDS: • resveratrol • antitumor activity • antimetastatic activity • angiogenesis • mice

Resveratrol (3,4',5-trihydroxystilbene) is a naturally occurring polyphenolic phytoalexin found in medicinal plants of the *Polygonum* species (Polygonaceae). In a previous report, we showed that resveratrol reduced lipogenesis from palmitate in the liver and adipose tissue in vivo (1). Furthermore, we reported that resveratrol strongly inhibited the formation of 5-lipoxygenase products, 5-hydroxy-6,8,11,14-eicosatetraenoic acid, leukotrienes B₄ and C₄, and the cyclooxygenase product thromboxane B₂ from arachidonic acid (2–4). We also showed the inhibitory effect of resveratrol on arachidonic acid-induced platelet aggregation (2). Resveratrol and its derivatives have been further shown to strongly inhibit the degranulation of human polymorphonuclear leukocytes (4). Recently, there have been a number of reports that resveratrol inhibits tumor growth and causes apoptosis as a cancer chemopreventive mechanism (5–12). Tumor angiogenesis involves the directional sprouting of new vessels toward a solid tumor. It should be noted that angiogenesis is no less impor-

tant to the growth of secondary tumor colonies than it is to the growth of primary solid tumors. Secondary tumors often give rise to tertiary tumors in distant organs by a process termed metastatic cascade (13–16). Previously, we reported that piceid (resveratrol-3-O-D-glucoside) inhibited the lung metastasis and the capillary-like network formation induced by Matrigel from human umbilical vein endothelial cells (HUVEC)² (17), and it has been suggested that piceid administered orally may be converted to an aglycone form of resveratrol by hydrolysis (17). The inhibition of distant metastasis to lung or liver from primary solid-tumors by resveratrol is as yet unproven. In the present study, we examined the effects of resveratrol isolated from *Polygonum cuspidatum* root on tumor growth and lung metastasis in mice bearing highly metastatic

² Abbreviations used: BAEC, bovine aorta endothelial cell; BCECF, 3'-O-acetyl-2',7'-bis(carboxyethyl)-4 or 5-carboxyfluorescein; DMEM, Dulbecco's modified Eagle's medium; EB, endothelial basal medium; EGM, endothelial basal medium supplemented growth factor; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; HUVEC, human umbilical vein endothelial cells; IC₅₀, 50% inhibitory concentration; LLC, Lewis lung carcinoma; NK, natural killer; NMR, nuclear magnetic resonance; PE, phycoerythrin; TCA, trichloroacetic acid; VEGF, vascular endothelial growth factor.

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Lewis lung carcinoma (LLC) tumors, and angiogenesis in tumor-induced *in vivo* and *in vitro* models.

MATERIALS AND METHODS

General experimental procedure. Melting points, determined using a Yamato MO-21 capillary apparatus (Yamato Science, Tokyo, Japan), are uncorrected. Infrared and UV spectra were measured using a Shimadzu IR-400 spectrometer (Shimadzu, Kyoto, Japan) and a JASCO ORD/UV-5 spectrometer (JASCO, Tokyo, Japan), respectively. The ^1H nuclear magnetic resonance (NMR) (4899.83 Hz) spectrum was recorded in DMSO- d_6 and solvent plus D_2O using a Varian Unity Inova 500 spectrometer (TOSO, Tokyo, Japan). Column chromatography was performed using silica gel 60 (70–230 mesh, ASTM, Merck KGaA, Darmstadt, Germany) and polyamide (ICN Pharmaceuticals, Costa Mesa, CA).

Materials. Stilbene such as resveratrol (3,5,4'-trihydroxystilbene) and piceid (resveratrol-3-O-D-glucoside) were isolated from the roots of *P. cuspidatum* by the method described previously (18,19). Briefly, the dried crushed roots (1 kg) were extracted with methanol (3 L) three times under reflux, and the extract solution was removed by reduced pressure to give dark brown extracts (210 g). Methanol extract (200 g) was chromatographed on a silica gel column eluted with a mixture of chloroform/methanol (3:1 to 1:1, v/v) and polyamide column-eluted methanol to give resveratrol and piceid. Resveratrol and piceid were identified by direct comparison with authentic samples. Furthermore, resveratrol and piceid were identified as *trans* forms by the measurement of the UV and ^1H NMR spectra. Mouse lymphocyte separation medium (Lympholytes-Mouse) was purchased from Dainippon Pharmacy (Osaka, Japan). Fluorescein isothiocyanate (FITC)-labeled anti-mouse CD 4, CD8 and phycoerythrin (PE)-labeled anti-mouse natural killer (NK)1.1 were purchased from Serotec (Oxford, England). Propidium iodide, 3'-O-acetyl-2',7'-bis(carboxyethyl)-4 or 5-carboxylfluorescein (BCECF)-AM solution (1 mmol/L) and RNase A were purchased from Wako Pure Chemical (Osaka, Japan). Matrigel basement membrane matrix was obtained from Becton Dickinson Labware (Bedford, MA). [Methyl- ^3H]-thymidine (specific activity; 740 GBq/mmol) and ^{125}I -vascular endothelial growth factor (VEGF) (specific activity; 4.26 MBq/ μg) were purchased from NEN Life Science Products, (Boston, MA). Dulbecco's modified Eagle's medium (DMEM), endothelial basal medium (EBM) and CS-C medium kits were obtained from Nissui Pharmaceutical (Tokyo, Japan), Clonetic (San Diego, CA) and Cell Systems (Kirkland, WA), respectively, and used as culture media. Antibiotic and antimycotic solutions (100 \times) containing 10^6 U penicillin, 10 g streptomycin and 25 mg amphotericin B/L in 9 g/L NaCl were purchased from Sigma Chemical (St Louis, MO). Fetal bovine serum (FBS) was purchased from Gibco BRL (Auckland, New Zealand). The 6-, 12-, 24-, 48- and 96-well plates were purchased from Corning Glass Works (Corning, NY). Collagen (Type I)-coated 6- and 24-well plates were purchased from Toyobo Engineering (Osaka, Japan) and Sumitomo Bakelite (Tokyo, Japan), respectively.

Cells. The highly metastatic, drug-resistant mouse LLC cells were obtained from Riken Gene Bank (Tukuba, Japan) and maintained in DMEM supplemented with 100 mL/L FBS, penicillin (1 $\times 10^5$ U/L), streptomycin (100 mg/L) and amphotericin B (0.25 mg/L). HUVEC and bovine aorta endothelial cells (BAEC) were purchased from Clonetics (San Diego, CA) and Sannkou Junyaku (Tokyo, Japan), respectively; the HUVEC and BAEC were seeded onto collagen (Type I)-coated 24- or 6-well plates and maintained in Clonetics endothelial basal medium supplemented growth factor (EGM) or CS-C media.

Animals. Female C57BL/6 strain mice (5 wk old) were obtained from Clea Japan (Osaka, Japan). They were housed for 1 wk in a room maintained at $25 \pm 1^\circ\text{C}$ with 60% relative humidity and given free access to nonpurified diet [(per 100 g diet): water 8 g, crude carbohydrate 51.3 g, crude protein 24.6 g, crude lipid 5.6 g, crude fiber 3.1 g, mineral mixture 6.4 g and vitamin mixture 1 g; Oriental Yeast, Osaka, Japan] and water. The room was illuminated for 12 h/d starting at 0700 h. Animals were treated according to the ethical guidelines of the Animal Center, School of Medicine, Ehime University.

Measurement of tumor growth and metastasis to lung in LLC-bearing mice. Solid-type LLC was prepared by subcutaneous transplantation of 5×10^5 cells (0.5 mL) into the backs of C57BL/6 female mice on d 0. Resveratrol (0.6, 2.5 or 10 mg/kg body) was administered intraperitoneally once (at 0700 h) daily for 21 consecutive days, starting 12 h after implantation of the tumor cells. Untreated mice (normal) and LLC-implanted mice (control) were given distilled water alone on the same schedule. The tumor volume was determined every 2–3 d by direct measurement with calipers and calculated using the formula, $[\text{width}^2 (\text{mm}^2) \times \text{length} (\text{mm})]/2$. On d 22, the mice were killed by cervical dislocation, and the spleen, thymus and lung quickly removed and weighed. The metastases to the lung were counted using a stereoscopic microscope. The spleen tissues were gently teased to release cells by means of dissecting forceps in cold PBS (pH 7.4). The cell suspension (5 mL) was layered on Lympholytes-Mouse (5 mL) and centrifuged at $1500 \times g$ for 30 min to isolate the lymphocytes. The number of lymphocytes was measured using a Coulter Counter. The cell concentration was adjusted to 2×10^{10} cells/L, and the 10 μL of FITC-labeled anti-mouse CD4, CD8 or PE-labeled antimouse NK1.1. was added to 100 μL of the cell suspension. After incubation for 30 min at 4°C , the lymphocytes were rinsed three times with 1 mL of PBS and centrifuged at $700 \times g$ for 5 min. Subsequently, the CD4^+ , CD8^+ and NK1.1^+ T-cell populations were analyzed by flow cytometry using a FACS Calibur (Becton Dickinson, Mountain View, CA).

Measurement of DNA synthesis in LLC cells. LLC cells were placed in DMEM supplemented with 100 mL/L FBS at 1×10^4 cell/well in 24-well culture plates. After the cells were cultured overnight, the medium was changed to fresh DMEM with 100 mL/L FBS and cells were exposed to the indicated amounts of resveratrol for 20 h; then the medium was replaced with [^3H]thymidine (18.5 kBq = 0.5 μCi /well) in DMEM with 100 mL/L FBS. After further incubation for 4 h, the cells were washed twice with PBS, immersed in 1 mL of 50 g/L trichloroacetic acid (TCA) for 1 h at 4°C , washed twice with 50 g/L TCA and solubilized with 100 μL of 0.2 mmol/L NaOH containing 5 g/L Triton X-100. Thymidine incorporation into the cells was determined by liquid scintillation counting.

Measurement of apoptosis and the cell cycle in LLC cells. LLC cells were placed in DMEM containing 100 mL/L FBS at 1×10^6 cells/well in 6-well culture plates. After the cells were cultured overnight, the medium was changed to fresh medium and cells were exposed to the indicated amounts of resveratrol for 20 h. The detached (nonadherent) cells were collected, and the attached (adherent) cells were then dispersed by adding PBS (pH 7.4) containing 2.5 g/L trypsin and 1 mmol/L EDTA. Adherent and nonadherent cells were sedimented at 200 g for 10 min, washed with ice-cold PBS and fixed in ice-cold 700 mL/L methanol for 2 h at 4°C , and then further washed twice with ice-cold PBS. The fixed cells were incubated with 1 mL of 0.25 mg/L RNAase A for 1 h at 37°C , washed twice with PBS, stained with propidium iodide (final concentration of 50 mg/L), and subjected to apoptosis and cell cycle analysis by flow cytometry using a FACS Calibur.

Measurement of angiogenesis induced by tumor cells. *In vivo* angiogenesis was assayed by the dorsal air-sac method. Briefly, 3×10^6 cultured LLC cells were suspended in DMEM and packed into a round-shaped nitrocellulose membrane chamber with a diameter of 14 mm (pore size 0.45 μm ; Millipore, Bedford, MA) and implanted onto the dorsal air sac of female C57BL/6 mice on d 0. Resveratrol (0.6, 2.5 or 10 mg/kg) was administered intraperitoneally twice daily from d 1 to 5. The mice were killed on d 6, and skin hair in contact with the chamber was carefully shaved. The formation of new blood vessels in the subcutaneous region was photographed.

Measurement of DNA synthesis in BAEC and HUVEC. BAEC were placed in CS-C medium containing 100 mL/L FBS at 2×10^4 cell/well in collagen-coated 24-well culture plates. After the cells were cultured overnight, the medium was changed to fresh CS-C medium containing 100 mL/L FBS, and cells were exposed to the indicated amounts of stilbenes for 20 h. Then the medium was replaced with [^3H]thymidine (18.5 kBq = 0.5 μCi /well) in CS-C medium containing 10% FBS. After further incubation for 4 h, cells were washed twice with ice-cold PBS, and the thymidine incorporation was measured by the same methods described above for LLC

cells. HUVEC (1×10^4 cells per well) were seeded onto Matrigel (10 $\mu\text{g}/\text{well}$)-coated 96-well culture plates in CS-C medium containing 100 mL/L FBS. After the cells were cultured overnight, the medium was changed to fresh medium, and the cells were exposed to the indicated amounts of stilbenes for 20 h; thymidine incorporation was measured by the methods described above.

LLC cell adhesion to HUVEC. Confluent HUVEC monolayers (second passage) grown on collagen-coated 24-well culture plates were incubated with the indicated amounts of stilbenes for 6 h at 37°C in a humidified chamber containing 5% CO_2 in CS-C medium containing 100 mL/L FBS. After the incubation period, HUVEC monolayers were washed twice with CS-C medium containing 100 mL/L FBS, and then BCECF-labeled LLC cells (1×10^4 cells per well) were seeded onto HUVEC monolayers treated with stilbenes and incubated for 2 h. After the incubation period, HUVEC were gently washed three times with the above medium to remove non-adherent LLC cells. LLC cells that adhered to HUVEC were solubilized by adding 5 g/L Triton X-100 (1 mL), and the fluorescence of BCECF released from the LLC cells was measured by fluorimetry (FP-777, JASCO) with excitation at 500 nm and emission at 540 nm. LLC cell adherence to HUVEC monolayers is expressed throughout as the percentage of adherence, and the fluorescence released from the BCECF-labeled LLC cells seeded in each well of the 24-well plates was taken as 100% total fluorescence intensity.

Measurement of tube-like network formation from HUVEC induced by Matrigel. Matrigel (150 μL) was placed into each well of a 48-well culture plate at 4°C and allowed to polymerize by incubation for 1 h at 37°C. HUVEC (second passage, 2×10^4 cells) were seeded onto the Matrigel in 270 μL of DMEM supplemented with 200 mL/L FBS and incubated with the indicated amounts of resveratrol at 37°C for 24 h in a humidified 5% CO_2 atmosphere. Four different phase-contrast microscopic fields ($\times 40$ and $\times 100$ magnification) per well were photographed, and the photomicrograph images were stored in a computer. The total length of tube structures in each photograph ($\times 40$ magnification) was measured using Adobe Photoshop software (Adobe, Tokyo, Japan).

Binding of ^{125}I -labeled VEGF to HUVEC. HUVEC were cultured to subconfluency on collagen-coated 24-well culture plates. The HUVEC were incubated with the indicated amounts of resveratrol for 30 min at room temperature. Subsequently, ^{125}I -VEGF ($\sim 5.6 \times 10^4$ dpm; specific activity 4.26 MBq/ μg) was added and incubated for 3 h in a humidified 5% CO_2 atmosphere. After unbound radioligand was removed, the cells were solubilized in 10 g/L SDS. The amount of ^{125}I -VEGF bound to HUVEC was measured using a gamma counter (COBRA II, Packard, Meriden, CT).

Statistical analysis. All values are expressed as means \pm SEM. Data were analyzed by one-way ANOVA, and then differences among means were analyzed using Fisher's protected Least Significant Difference multiple comparison test. Differences were considered significant at $P < 0.05$.

RESULTS

Antitumor and antimetastatic activities. Tumor growth and final tumor weight were significantly inhibited by the intraperitoneally administered resveratrol at doses of 2.5 and 10 mg/kg, but 0.6 mg/kg of resveratrol had no effect (Fig. 1A and B). Resveratrol (2.5 and 10 mg/kg) significantly reduced the number of tumor cell colonies that metastasized to the lung compared with the LLC-bearing mice (Fig. 2).

Body weight, spleen and thymus weight, and the numbers of splenic lymphocytes, CD4^+ , CD8^+ and NK1.1^+ T cells. Resveratrol had no effect on either initial or final body weight in LLC-bearing mice compared with that in normal mice (Table 1). In LLC-bearing mice, the spleen weight was significantly greater than that of the normal mice. In contrast, thymus weight of LLC-bearing mice was significantly lower than that of normal mice. Resveratrol tended to prevent the increase of spleen weight and the reduction of thymus weight in LLC-bearing mice (Table 1). The number of lymphocytes in the spleens of LLC-bearing mice significantly increased to

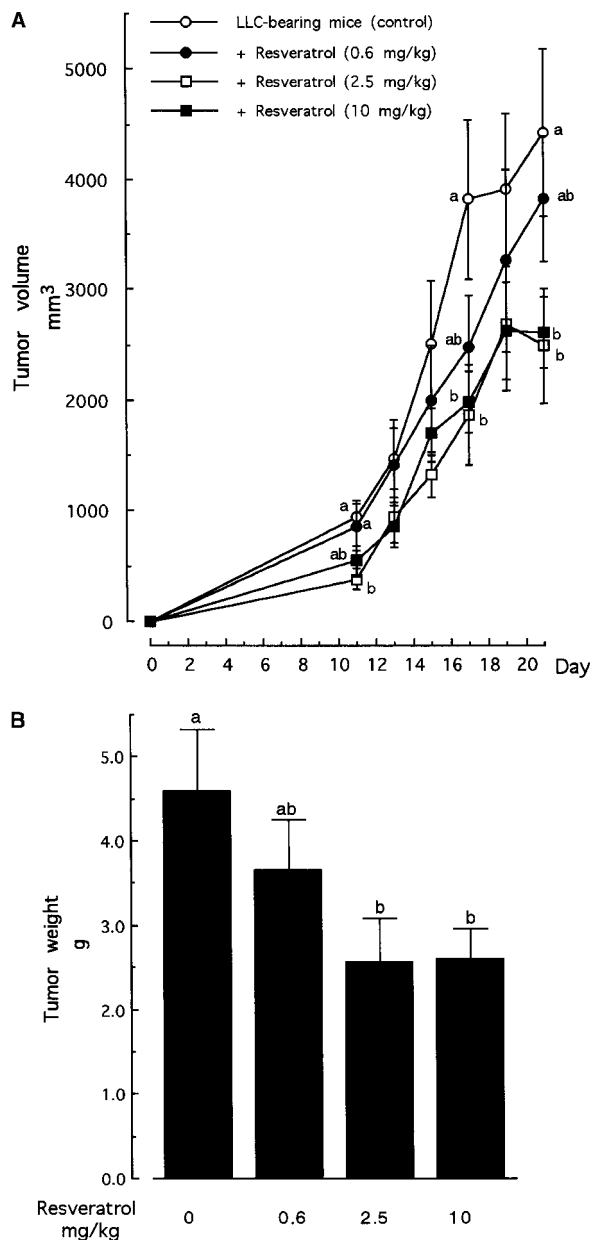


FIGURE 1 Effects of resveratrol on tumor volume (A) over 21 d and final tumor weight (B) on d 22 in Lewis lung carcinoma (LLC)-bearing mice. Solid-type LLC was prepared by subcutaneous transplantation of 5×10^5 cells (0.5 mL) into the backs of C57BL/6 female mice on d 0. Resveratrol (0.6, 2.5 or 10 mg/kg body) was administered intraperitoneally once (at 0700 h) daily for 21 consecutive days, starting 12 h after implantation of the tumor cells. Untreated mice (normal) and LLC-implanted mice (control) were given distilled water alone on the same schedule. The tumor volume was determined every 2–3 d by direct measurement with calipers and calculated using the formula, $[\text{width}^2 (\text{mm}^2) \times \text{length} (\text{mm})]/2$. Values are means \pm SEM, $n = 7$. Those not sharing a letter are significantly different, $P < 0.05$.

$7.29 \pm 0.74 \times 10^6$ from $2.90 \pm 0.33 \times 10^6$ in normal mice. In contrast, the splenic CD4^+ and CD8^+ cells in LLC-bearing mice were significantly reduced to 7.69 ± 0.85 and $14.1 \pm 1.65\%$ from 22.5 ± 0.97 and $42.9 \pm 3.29\%$ in normal mice, respectively. NK1.1^+ T cells were not affected. The increase in splenic lymphocyte and reductions of CD4^+ and CD8^+ T cells were not prevented by the intraperitoneally administered resveratrol (data not shown).

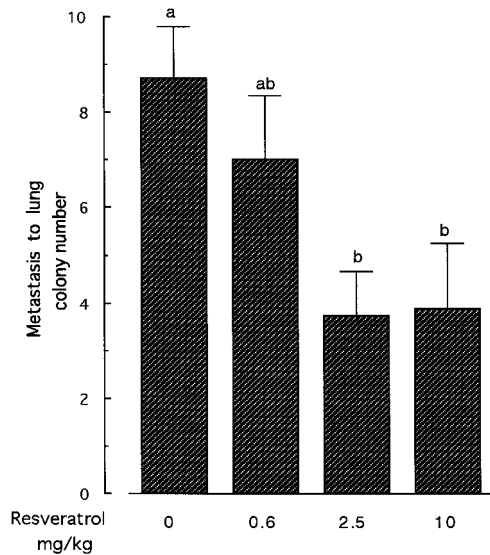


FIGURE 2 Effects of resveratrol on the numbers of colonies of Lewis lung carcinoma (LLC) cells metastasizing to the lung on d 22 in LLC-bearing mice. Values are means \pm SEM, $n = 7$. Those not sharing a letter are significantly different, $P < 0.05$.

DNA synthesis, apoptosis and the cell cycle in LLC cells (in vitro). Resveratrol inhibited the DNA synthesis in LLC cells with a 50% inhibitory concentration (IC_{50}) of 6.8 $\mu\text{mol/L}$ (Fig. 3). Treatment with 100 $\mu\text{mol/L}$ resveratrol for 24 h increased apoptosis to 20.6 ± 1.35 from $12.1 \pm 0.36\%$ in LLC cells (Table 2). In addition, resveratrol decreased the S phase population at concentrations of 50 and 100 $\mu\text{mol/L}$. The proportion of LLC cells in the G_2/M phase of the cell cycle was increased by the treatment with 50 $\mu\text{mol/L}$ resveratrol.

Tumor- and Matrigel-induced angiogenesis and (in vivo and in vitro). Five days after implantation of the LLC cells, which were packed into the membrane chamber, neovascularization was evident in the region in contact with the chamber containing LLC cells. The intraperitoneally administered resveratrol (2.5 and 10 mg/kg twice per day) prevented the neovascularization induced by LLC cells (Fig. 4). HUVEC formed capillary-like networks on Matrigel 24 h after seeding. Resveratrol dose dependently inhibited angiogenesis of HUVEC at 5–100 $\mu\text{mol/L}$ concentrations (Fig. 5).

DNA synthesis in BAEC and HUVEC (in vitro). Resveratrol significantly inhibited DNA synthesis of BAEC at concentrations of 50–500 $\mu\text{mol/L}$, but not at 5 and 10 $\mu\text{mol/L}$ (data not shown). The inhibition ratios were 45.5% at 50 $\mu\text{mol/L}$, 81.5% at 100 $\mu\text{mol/L}$ and 68.4% at 500 $\mu\text{mol/L}$,

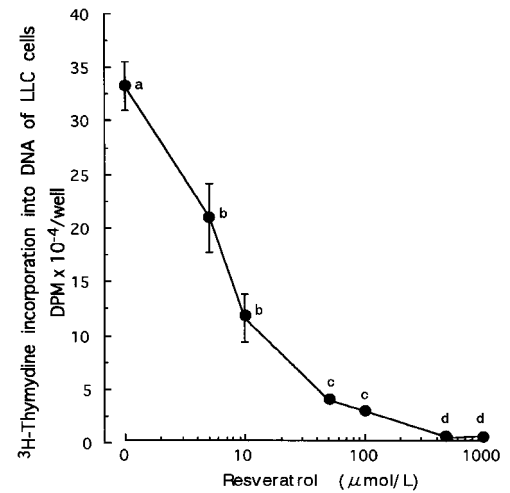


FIGURE 3 Effects of resveratrol on ^3H -thymidine incorporation into DNA of Lewis lung carcinoma (LLC) cells in mice. Values are means \pm SEM, $n = 4$. Those not sharing a letter are significantly different, $P < 0.05$.

respectively, with an IC_{50} of 54 $\mu\text{mol/L}$. Furthermore, resveratrol inhibited DNA synthesis of HUVEC at 100 $\mu\text{mol/L}$; its inhibition ratio was 69.3%, but not at concentrations of 5, 10 and 50 $\mu\text{mol/L}$; the IC_{50} was 89 $\mu\text{mol/L}$ (data not shown).

Adherence of LLC cells and binding of ^{125}I -VEGF to HUVEC (in vitro). When LLC cells were seeded onto the HUVEC monolayer, the adherence of LLC cells to HUVEC was increased from 10.4 ± 2.8 to $50.8 \pm 6.4\%$ of total cells. Resveratrol had no effect on adherence of LLC cells to HUVEC (data not shown). Resveratrol significantly inhibited the binding of VEGF to HUVEC at 10–100 $\mu\text{mol/L}$ concentrations (Fig. 6).

DISCUSSION

The removal of certain tumors, for example, breast carcinoma, colon carcinoma and osteogenic sarcoma, may be followed by the rapid growth of distant metastases to organs such as lung, liver or bone. Therefore, it is necessary to develop new anticancer agents with antitumor and antimetastatic activities. The roots of *P. cuspidatum* are traditionally used as a remedy or prevention for allergic inflammatory diseases, hyperlipidemia and cancer. Although resveratrol is reported to contain a cancer chemopreventive agent (5,6), the inhibitory action by resveratrol on distant metastases to other organs from primary solid-tumors is as yet unproven. In this study, we

TABLE 1

Effects of resveratrol on the weights of body, spleen and thymus in Lewis lung carcinoma (LLC)-bearing mice¹

	<i>n</i>	Initial body	Final body	Spleen	Thymus
		<i>g</i>		<i>mg</i>	
Normal	5	18.2 \pm 0.47	20.1 \pm 0.79	119.6 \pm 9.9 ^b	54.1 \pm 7.6 ^b
LLC-bearing mice + resveratrol	7	18.1 \pm 0.95	20.6 \pm 0.48	351.1 \pm 21.7 ^a	23.5 \pm 5.64 ^a
(0.6 mg/kg)	7	18.8 \pm 0.15	21.6 \pm 0.90	298.3 \pm 17.0 ^a	34.4 \pm 6.54 ^a
(2.5 mg/kg)	7	18.5 \pm 0.41	20.9 \pm 0.51	267.7 \pm 33.0 ^c	45.8 \pm 7.79 ^b
(10 mg/kg)	7	18.9 \pm 0.28	21.5 \pm 0.37	251.1 \pm 32.8 ^c	40.5 \pm 4.68 ^{ab}

¹ Values are means \pm SEM. Those with superscripts not sharing a letter are significantly different, $P < 0.05$.

TABLE 2

Effects of resveratrol on apoptosis, G₀/G₁, S and G₂/M phases of cell cycle in Lewis lung carcinoma cells¹

Resveratrol $\mu\text{mol/L}$	Apoptosis	G ₀ /G ₁	S	G ₂ /M
	% total cells			
0	12.1 ± 0.36 ^a	50.0 ± 1.97	35.2 ± 1.72 ^a	14.8 ± 0.29 ^b
5	9.43 ± 0.51 ^a	44.6 ± 0.75	37.9 ± 1.25 ^a	17.5 ± 0.49 ^b
10	9.65 ± 0.61 ^a	39.9 ± 1.54	43.8 ± 2.12 ^a	16.3 ± 0.70 ^b
50	12.5 ± 0.97 ^a	46.8 ± 1.15	22.1 ± 1.03 ^b	31.1 ± 0.26 ^a
100	20.6 ± 1.35 ^b	52.9 ± 1.16	29.2 ± 0.27 ^b	17.8 ± 1.42 ^b

¹ Values are means ± SEM, *n* = 3. Those with superscripts not sharing a letter are significantly different, *P* < 0.05.

examined the effects of resveratrol contained in medicinal plants on tumor growth and metastasis to the lung using mice bearing the highly metastatic, drug-resistant mouse tumor, LLC. It has been reported that LLC-bearing C57BL/6 mice had lung metastasis in addition to tumor growth (20–23). We found that tumor growth and lung metastasis were inhibited by the intraperitoneal administration of resveratrol [2.5 and 10 mg/(kg · d)] for 21 consecutive days in LLC-bearing C57BL/6 mice. Mu et al. (24) and Brunda et al. (25) reported that metastases to distant organs and tumor growth were prevented through natural killer cell and cytotoxic T lymphocyte activation by the administration of interleukin-12. Because the intraperitoneal administration of resveratrol did not affect the numbers of CD4⁺, CD8⁺ T cells or NK1.1⁺ T cells in the spleens of LLC-bearing mice, the inhibitory effects of resveratrol on tumor growth and lung metastasis could not be explained by natural killer and cytotoxic T lymphocyte activation. Next, we examined the effects of resveratrol on the DNA synthesis of LLC cells. The results showed that resveratrol inhibited the DNA synthesis with an IC₅₀ of 6.8 $\mu\text{mol/L}$. Resveratrol at 100 $\mu\text{mol/L}$ caused apoptosis, decreased the S phase population at 50 and 100 $\mu\text{mol/L}$ and increased the G₂/M phase at 50 $\mu\text{mol/L}$ in LLC cells. Ragion et al. (7) reported that 30 $\mu\text{mol/L}$ resveratrol arrested the cell division cycle at the S/G₂ phase transition in the promyelocytic cell line HL-60. Clément et al. (11) reported that the cell death induced by 32 $\mu\text{mol/L}$ resveratrol in HL 60 cells is tumor specific, and reported findings supporting the involvement of the Fas-Fas ligand system as the apoptotic trigger. These findings suggest that resveratrol may contribute to the inhibition

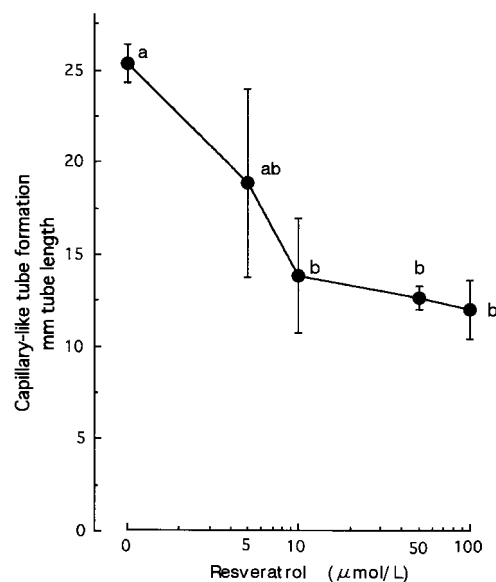


FIGURE 5 Effects of resveratrol on Matrigel-induced capillary-like tube formation from human umbilical vein endothelial cells (HUVEC). Values are means ± SEM, *n* = 4. Those not sharing a letter are significantly different, *P* < 0.05.

of primary tumor growth and metastasis to distant organs through the inhibition of DNA synthesis of tumor cells by resveratrol. However, the discrepancy between apoptotic and DNA synthesis inhibitory concentrations of resveratrol are unknown; therefore, further studies are warranted.

Tumor cell interactions with platelets, endothelial cells and the subendothelial matrix are considered essential intermediate steps for the completion of the metastatic cascade for tumors (14,26–28). To test the antimetastatic activity of resveratrol, we examined the effects of resveratrol on the interactions of LLC cells with HUVEC. Resveratrol had no effect on the adherence of LLC cells to monolayer HUVEC (data not shown), indicating that the antimetastatic activities of resveratrol are not due to inhibition of the adherence of LLC cells to HUVEC.

Angiogenesis is the growth of new capillary blood vessels from preexisting capillaries and postcapillary venules. The solid tumors cause neovascularization, and the resultant angiogenesis from solid tumors stimulates growth and metastasis (14,15,22,29–33). Resveratrol inhibited tumor-induced neo-

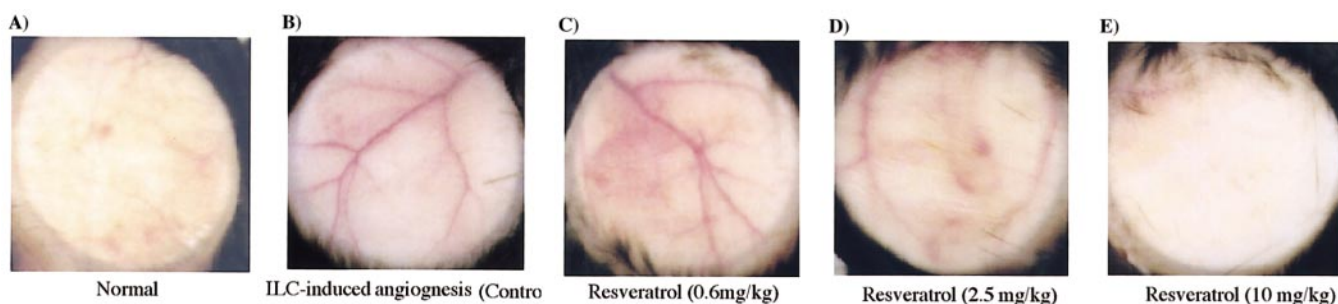


FIGURE 4 Photographs of the inhibition of Lewis lung carcinoma (LLC)-induced neovascularization by resveratrol in mice. Chambers packed with LLC cells were subcutaneously implanted into a dorsal air-sac of C57BL/6 mice on d 0. Dulbecco's modified Eagle's medium alone (normal, A), LLC packed chamber (control, B) or 0.6 mg/kg (C), 2.5 mg/kg (D) and 10 mg/kg (E) of resveratrol was administered intraperitoneally from d 1 to 5. The mice were killed on d 6, and skin hair in contact with the chamber was carefully shaved. The formation of new blood vessels in the subcutaneous region was photographed.

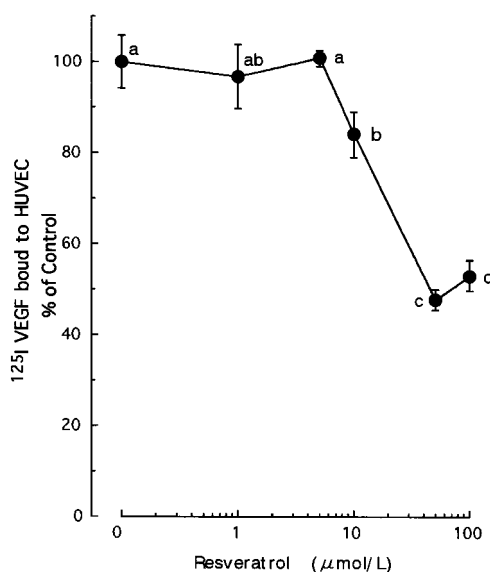


FIGURE 6 Effects of resveratrol on the binding of ^{125}I -vascular endothelial growth factor (VEGF) to human umbilical vein endothelial cells (HUVEC). Values are means \pm SEM, $n = 4$. Those not sharing a letter are significantly different, $P < 0.05$.

vascularization (in vivo) and capillary-like network tube formation of HUVEC (in vitro). But resveratrol also had no effect on DNA synthesis of BAEC and HUVEC at the same concentrations. Tumor cells are thought to secrete angiogenic factor(s) that induce neovascularization around the tumors (34–36). VEGF is a secretory angiogenic factor (37). Asano et al. (38) reported that monoclonal antibody to VEGF prevented the tumor growth and tumor-induced neovascularization. Resveratrol inhibited the binding of VEGF to HUVEC at concentrations of 10–100 $\mu\text{mol/L}$. In summary, the inhibition ratios for DNA synthesis of LLC cells, capillary-like network formation DNA syntheses of BAEC and HUVEC, and VEGF binding to HUVEC by resveratrol at 10 $\mu\text{mol/L}$ were 65.6, 45.5, -7.1 , -21.3 and 15.8%, respectively. Therefore, these findings suggest that the mechanism of antitumor and antimetastatic actions of resveratrol might be due to the inhibition of DNA synthesis in LLC cells and the inhibition of tumor-induced neovascularization through the inhibition of capillary-like network formation from HUVEC.

LITERATURE CITED

- Arichi, H., Kimura, Y., Okuda, H., Baba, K., Kozawa, M. & Arichi, S. (1982) Effects of stilbene components of the roots of *Polygonum cuspidatum* Sieb. et Zucc. on lipid metabolism. *Chem. Pharm. Bull.* 30: 1766–1770.
- Kimura, Y., Okuda, H. & Arichi, S. (1985) Effects of stilbenes on arachidonate metabolism in leukocytes. *Biochim. Biophys. Acta* 834: 275–278.
- Kimura, Y., Okuda, H. & Arichi, S. (1985) Effects of stilbene derivatives on arachidonate metabolism in leukocytes. *Biochim. Biophys. Acta* 837: 209–212.
- Kimura, Y., Okuda, H. & Kubo, M. (1995) Effects of stilbenes isolated from medicinal plants on arachidonate metabolism and degranulation in human polymorphonuclear leukocytes. *J. Ethnopharmacol.* 45: 131–139.
- Jang, M., Cai, L., Udeani, G. O., Slowing, K. V., Thomas, C. F., Beecher, C. W. W., Fong, H. H. S., Farnsworth, N. R., Kinghorn, A. D., Mehta, R. G., Moon, R. C. & Pezzuto, J. M. (1997) Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science* (Washington, DC) 275: 218–220.
- Ciolino, H. P., Daschner, P. J. & Yeh, G. C. (1998) Resveratrol inhibits transcription of CYP1A1 in vitro by preventing activation of the aryl hydrocarbon receptor. *Cancer Res.* 58: 5707–5712.
- Ragione, F. D., Cucciolla, V., Borriello, A., Pietra, V. D., Racioppi, L., Soldati, G., Hanna, C., Galletti, P. & Zappia, V. (1998) Resveratrol arrests the cell division cycle at S/G₂ phase transition. *Biochem. Biophys. Res. Commun.* 250: 53–58.

- Fontecave, M., Lepoivre, M., Elleingand, E., Geréz, C. & Guittelet, O. (1998) Resveratrol, a remarkable inhibitor of ribonucleotide reductase. *FEBS Lett.* 421: 277–279.
- Subbaranmaiah, K., Chung, W. J., Michaluart, P., Telang, N., Tanabe, T., Inoue, H., Jang, M., Pezzuto, J. M. & Dannenberg, A. J. (1998) Resveratrol inhibits cyclooxygenase-2 transcription and activity in phorbol ester-treated human mammary epithelial cell. *J. Biol. Chem.* 273: 21875–21882.
- Sun, N. J., Woo, S. H., Cassady, J. M. & Snapka, R. M. (1998) DNA polymerase and topoisomerase II inhibitors from *Psoralea corylifolia*. *J. Nat. Prod.* 61: 362–366.
- Clément, M.-V., Hirpara, J. L., Chawdhury, S.-H. & Pervaiz, S. (1998) Chemopreventive agent resveratrol, a natural product derived from grapes, triggers CD95 signaling-dependent apoptosis in human tumor cells. *Blood* 92: 996–1002.
- Carbó, N., Costelli, P., Baccino, F. M., López-Soriano, F. J. & Argilés, J. M. (1999) Resveratrol, a natural product present in wine, decreases tumor growth in rat tumor model. *Biochem. Biophys. Res. Commun.* 254: 739–743.
- Weiss, L., Orr, F. W. & Honn, K. V. (1988) Interactions of cancer cells with the microvasculature during metastasis. *FASEB J.* 2: 12–21.
- Blood, C. H. & Zetter, B. R. (1990) Tumor interactions with the vasculature: angiogenesis and tumor metastasis. *Biochim. Biophys. Acta* 1032: 89–118.
- Ferrara, N. (1995) Missing link in angiogenesis. *Nature* (Lond.) 376: 467 (Abst).
- Kerbel, R. S. (1997) A cancer therapy resistant to resistance. *Nature* (Lond.) 390: 335–336.
- Kimura, Y. & Okuda, H. (2000) Effects of naturally occurring stilbene glucosides from medicinal plants and wine, on tumor growth and lung metastasis in Lewis lung carcinoma-bearing mice. *J. Pharm. Pharmacol.* 52: 1287–1295.
- Kubo, M., Kimura, Y., Shin, H., Haneda, T., Tani, T. & Namba, K. (1981) Studies on the antifungal substance of crude drug (II). On the roots of *Polygonum cuspidatum* Sieb. et Zucc. (Polygonaceae). *Shoyakugaku Zasshi* 35: 58–64.
- Kimura, Y., Kozawa, M., Baba, K. & Hata, K. (1983) New constituents of roots of *Polygonum cuspidatum*. *Planta Med.* 48: 164–169.
- DeWys, W. D. (1972) Studies correlating the growth rate of a tumor and its metastases and providing evidence for tumor-related systemic growth-retarding factor. *Cancer Res.* 32: 374–379.
- Gorelik, E., Segal, S. & Feldman, M. (1978) Growth of local tumor exerts a specific inhibitory effect on progression of lung metastases. *Int. J. Cancer* 21: 617–625.
- Gorelik, E., Segal, S. & Feldman, M. (1980) Control of lung metastasis progression in mice: role of growth kinetics of 3 LL Lewis lung carcinoma and host immune reactivity. *J. Natl. Cancer Inst.* 65: 1257–1264.
- O'Reilly, M. S., Hongren, L., Shing, Y., Chen, C., Rosenthal, R. A., Moses, M., Lane, W. S., Cao, Y., Sage, E. H. & Folkman, J. (1994) Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. *Cell* 79: 315–328.
- Mu, J., Zou, J.-P., Yamamoto, N., Tsutsui, T., Tai, X.-G., Kobayashi, M., Hermann, S., Fujiwara, H. & Hamaoka, T. (1995) Administration of recombinant interleukin 12 prevents outgrowth of tumor cells metastasizing spontaneously to lung and lymph nodes. *Cancer Res.* 55: 4404–4408.
- Brunda, M. J., Luistro, L., Warrier, R. R., Wright, R. B., Hunnard, B. R., Murphy, M., Wolf, S. F. & Gately, M. K. (1993) Antitumor and antimetastatic activity of interleukin-12 against murine tumors. *J. Exp. Med.* 178: 1223–1230.
- Weiss, L. (1976) In: *Fundamental Aspects of Metastasis* (Weiss, L., ed.), pp. 51–70, North Holland, Amsterdam, The Netherlands.
- Liotta, L. A. (1986) Tumor invasion and metastases. Role of the extracellular matrix. *Cancer Res.* 46: 1–7.
- Honn, K. V. & Tang, D. G. (1992) Adhesion molecules and tumor cell interaction with endothelium and subendothelial matrix. *Cancer Metastasis Rev.* 11: 353–375.
- Folkman, J. (1995) Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat. Med.* 1: 27–31.
- Koch, A. E., Halloran, M. M., Haskell, C. J., Shah, M. R. & Polverini, P. J. (1995) Angiogenesis mediated by soluble forms of E-selectin and vascular cell adhesion molecule-1. *Nature* (Lond.) 376: 517–519.
- Bischoff, J. (1997) Cell adhesion in vascular biology: cell adhesion and angiogenesis. *J. Clin. Invest. (suppl.)* 100: s37–s39.
- O'Reilly, M. S., Boem, T., Shing, Y., Fukai, N., Vasios, G., Lane, W. S., Flynn, E., Birkhead, J. R., Olsen, B. R. & Folkman, J. (1997) Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. *Cell* 88: 277–285.
- Boem, T., Folkman, J., Browder, T. & O'Reilly, M. S. (1997) Antiangiogenic therapy of experimental cancer does not induce acquired drug resistance. *Nature* (Lond.) 390: 404–407.
- Folkman, J. & Klagsbrum, M. (1987) Angiogenic factors. *Science* (Washington, DC) 235: 442–447.
- Klagsbrum, M. & D'Amore, P. A. (1991) Regulators of angiogenesis. *Annu. Rev. Physiol.* 53: 217–239.
- Folkman, J. & Shing, Y. (1992) Angiogenesis. *J. Biol. Chem.* 267: 10931–10934.
- Leung, D. W., Cachianes, G., Kuang, W.-J., Goedel, D. V. & Ferrara, N. (1989) Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science* (Washington, DC) 246: 1306–1309.
- Asano, M., Yukita, A. & Suzuki, H. (1999) Wide spectrum of antitumor activity a neutralizing monoclonal antibody to human vascular endothelial growth factor. *Jpn. J. Cancer Res.* 90: 93–100.