Resveratrol Suppresses the Angiogenesis and Tumor Growth of Gliomas in Rats

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ABSTRACT

Purpose: We wanted to investigate the antitumor effects and effect on angiogenesis of resveratrol in rat RT-2 gliomas.

Experimental Design: RT-2 glioma cells were treated with resveratrol, and then cytotoxicity was assayed, apoptosis was measured by flow-activated cell sorter flow cytometry, and expression of vascular endothelial growth factor was measured by reverse transcription-PCR. Tumor size, animal survival time, and survival rate were followed in resveratrol-treated rats with s.c. or intracerebral gliomas. Furthermore, in vitro proliferation was assayed to explore the effect of resveratrol on the proliferation of ECV304 human umbilical vein endothelial cells. Expression of CD31 in resveratrol-treated gliomas was followed immunohistochemically to study the effect of resveratrol on the glioma-induced angiogenesis.

Results: Resveratrol was demonstrated to exert cytotoxic effects and induce glioma cell apoptosis in a concentration- and time-dependent manner (P < 0.05). Resveratrol (40 mg/kg/day) exerted significant antitumor effects on s.c. tumors, including slower tumor growth rate, longer animal survival time, and higher animal survival rate (P < 0.05). In contrast, resveratrol affected intracerebral tumors at only an increased dose (100 mg/kg/day), prolonging animal survival (P < 0.05) without affecting survival rate. The expression of vascular endothelial growth factor in the glioma cells and the proliferation of ECV304 cells were inhibited by resveratrol in a concentration-dependent manner. Immunohistochemical analyses showed that the s.c. gliomas from resveratrol-treated rats had fewer microvessel densities than did control rats (P < 0.01).

Conclusions: Resveratrol caused significant glioma cell cytotoxicity and apoptosis, exerted antitumor effects on the s.c. and intracerebral gliomas, and inhibited angiogenesis in s.c. gliomas. Thus, resveratrol might be considered a possible treatment strategy for gliomas.

INTRODUCTION

The prognosis of patients with malignant glioma, the most common primary brain tumor, is quite poor (1–6). Even using multidisciplinary treatment strategies including surgery, radiotherapy, and chemotherapy, the life expectancy of patients with malignant gliomas is usually <1 year from the time of diagnosis, and the 5-year survival rate is <5.5% (1–6). Therefore, developing a better therapeutic strategy for malignant brain tumors is mandatory.

Resveratrol (3,4,5′-trihydroxy-trans-stilbene, C14H12O3, molecular weight 228.2) is a natural polyphenol. The source of resveratrol is mainly from grapes and mulberries (7). Resveratrol has been found to act as a strong antioxidant (8) and can reduce the oxidation of lipoprotein (9) and the synthesis of lipid in the liver (10). Resveratrol can also protect the vessels from arteriosclerosis (8, 9), inhibit platelet aggregation, and reduce the synthesis of eicosanoid (7). In recent years, resveratrol was further demonstrated to be an antitumor and chemopreventive agent and found to affect cellular proliferation through its action on tumor initiation, promotion, and progression (11, 12). Resveratrol has been found to inhibit the proliferation of several kinds of tumors such as leukemia, prostate, breast, and colon cancers (13–17). The mechanisms of the antitumor effects of resveratrol are not fully understood, although some mechanisms have been proposed. Resveratrol has antiestrogentic activity (15); activates the expression of p53 (18), Fas-Fas ligand system (13), and mitogen-activated protein kinase (MAPK) (19, 20); inhibits p4501A1 (21), ribonucleotide reductase (22), ornithine decarboxylase (16), protein kinase C (PKC) (23), DNA polymerase (24), cyclo-oxygenase (23, 25), and cell cycle progression (16, 26); and induces cellular apoptosis (13, 17, 18, 20, 27). Recently, resveratrol was further demonstrated to inhibit angiogenesis, suppress capillary-like tube formation of human umbilical vein endothelial cells (HUVECs), and decrease angiogenesis in mouse lung cancer (28).

As mentioned above, resveratrol is a PKC inhibitor and has an anti-angiogenesis effect (23, 28). PKC is important in the regulation of the growth of glioma cells (29, 30) in that glioma cells have high expression of PKC, and increased activity of PKC is correlated with glioma cell proliferation (30). Furthermore, inhibitors of the signaling pathway of PKC suppress the proliferation and induce apoptosis of glioma cells (29, 30). In addition, angiogenesis in malignant glioma is very prominent, and the vessel number in the tumor is correlated with the degree
of malignancy (31, 32). Therefore, both PKC and angiogenesis have intimate relationships with gliomas, and resveratrol (an inhibitor of both PKC and angiogenesis) thus might be used to treat gliomas. In the literature, there is no report on the effect of resveratrol on gliomas, and only a few reports on the effect of resveratrol on the angiogenesis of cancer. Thus, in this study, we investigated the effects of resveratrol on the proliferation and apoptosis of glioma cells, the in vitro antitumor effects of resveratrol on gliomas, and the effect of resveratrol on angiogenesis in gliomas. Defective control of apoptosis has been considered to play a central role in tumor pathogenesis (33), and resveratrol has been found to induce apoptosis in a variety of cancer cells (13, 17, 18, 20, 27). Furthermore, resveratrol has been found to activate the MAPK signaling pathway, one of four signal transduction systems used by mammalian (34), mouse epidermal, and human neuroblastoma cells (19, 20), and such activation of MAPK is related to apoptosis in cancer cells (19, 20, 35–39). Thus, we also studied the effect of resveratrol on the expression of MAPK in the gliomas.

MATERIALS AND METHODS

Cell Lines and Cell Culture. The cell lines used in this study were the rat RT-2 glioma cell line and ECV304 human umbilical vein endothelial cell line. RT-2 is derived from an avian sarcoma virus-induced brain tumor in the Fischer 344 rat (40). ECV304 is an immortalized HUVEC line (41). Both RT-2 and ECV304 cells were maintained in DMEM (Seromed, Berlin, Germany) supplemented with 10% FCS (Biological Industries, Kibbutz Beth Haemek, Israel), 2 mM l-glutamate, 100 units/ml penicillin, and 100 µg/ml streptomycin. All cells were cultured at 37°C in a 5% CO2 incubator.

Cytotoxicity Effects of Resveratrol on the RT-2 Glioma Cells. The sensitivity of the RT-2 cells to resveratrol (Sigma Chemical Co., St. Louis, MO) was determined in vitro by an 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT)-based colorimetric assay (42). For this purpose, 5 × 103 RT-2 cells were seeded in triplicate wells of a flat-bottomed 24-well microtiter plate and cultured overnight before resveratrol treatment. The RT-2 cells were exposed to 0, 0.1, 1, 5, 10, 25, 100, 250, and 500 µM resveratrol (dissolved in DMSO; Sigma; Refs. 17, 28) for 6, 24, or 48 h. The cells were incubated for a total of 5 days subsequent to cell seeding. The extent of the cell proliferation and cell viability was then determined by MTT assay. The particular resveratrol concentration at which 50% of the RT-2 cells were killed was designated the IC50 (i.e., 50% inhibitory concentration). The doses of resveratrol for subsequent experiments were selected according to the results of the cytotoxicity assay.

Analysis of Resveratrol-induced Apoptosis by Flow-activated Cell Sorter (FACS) Flow Cytometry. The apoptosis fraction of the glioma cells after exposure to resveratrol was investigated using FACSscan analyses. Briefly, following treatment with 0, 0.1, 1, 5, 10, 25, or 100 µM resveratrol for 6, 24, or 48 h, 106 RT-2 cells were trypsinized and washed with PBS twice. After this, the cells were stored in one milliliter of 80% ethanol/PBS at −20°C for subsequent analysis. For FACSscan analysis, the cells were centrifuged at 14,000 rpm for 5 min, washed with PBS twice, incubated with 0.5 ml of 0.5% Triton X-100/PBS and 5 µg of RNase A for 30 min, stained with 0.5 ml of 50 µg/ml propidium iodide/PBS in the dark, and finally analyzed using a FACScan flow cytometry system (FACSCalibur; Becton Dickinson Immunocytometry System, San Jose, CA).

Resveratrol Treatment of the s.c. Gliomas in Rats. Fischer 344 rats weighing 200–350 g were used for experiments, with 10 rats in each group. S.c. tumors were induced by injecting 1 × 105 RT-2 glioma cells (in 10 µl of PBS) into the right flank of the rats, and these rats received various treatments starting immediately (small s.c. glioma model) or at day 5 (large s.c. glioma model) after tumor cell inoculation. The experiment for the study of the effect of resveratrol on the small s.c. gliomas consisted of four groups. Group A received no treatment. Groups B, C, and D were treated with i.p. injection of propylene glycol (vehicle, 0.5 ml), 10 mg/kg of resveratrol (in 0.5 ml of propylene glycol), or 40 mg/kg of resveratrol (in 0.5 ml of propylene glycol), once daily for 4 weeks, respectively (43, 44). The experiment for the study of the effect of resveratrol on the large s.c. gliomas consisted of three groups: Group A-1 received no treatment; groups B-1 and D-1 were treated with i.p. injection of propylene glycol (vehicle, 0.5 ml) or 40 mg/kg resveratrol (in 0.5 ml of propylene glycol), once daily for 4 weeks, respectively. The animal survival time and survival rate were then followed. Survival was monitored over a period of 150 days. Survival rates and survival times were compared among these groups.

Observation of Tumor Growth Rates. The growth rates of the s.c. tumors described above were monitored. Tumor size was measured twice weekly until the rat died. A blinded observer measured tumor length and width. The volume of the tumor was calculated from the formula V = 1/2 (d1 × d2 × d3), where d1, d2, and d3 were tumor diameters measured with calipers in mutually perpendicular directions (45). Average daily tumor volumes from each group were compared throughout the course of the experiment. Group averages were not compared after one or more animals in the group died.

Resveratrol Treatment of Intracerebral Gliomas in Rats. Intracerebral tumors were induced by implanting tumor cells into the brains of Fischer 344 rats (10 rats in each group) by stereotactic surgery. The rats were anesthetized by 10 mg/kg of xylazine and 80 mg/kg of ketamine hydrochloride in the following experiments. Each rat was fixed in a stereotactic frame, a burr hole was drilled, and tumor cells were injected into the right caudate-putamen (coordinates: 2.5 mm lateral, 1 mm anterior to the bregma, 4 mm below the dura) via a Hamilton syringe. Typically, 5 × 105 tumor cells were suspended in 5 µl of PBS. The injection was accomplished in 3 min, with the syringe remaining in place for 3 min; it was then slowly withdrawn for another 3 min. These rats received various treatments starting immediately (small intracerebral glioma model) or at day 3 (large intracerebral glioma model) after tumor cell inoculation. The treatment was continued until a 4-week treatment was completed or the animal died, whichever came first. The experiment for the study of the effect of resveratrol on the small intracerebral gliomas consisted of five groups. Group E received no treatment. Groups F, G, H, and I were treated with i.p. injection of propylene glycol (vehicle, 0.5 ml), 10 mg/kg of resveratrol (in 0.5 ml of propylene glycol), 40 mg/kg resveratrol
Cytotoxicity effects of resveratrol on the RT-2 glioma cells. In total, $5 \times 10^4$ RT-2 cells were seeded in triplicate wells in flat-bottomed, 24-well microtiter plates. Subsequently, the cells were exposed to 0, 0.1, 1, 5, 10, 25, 100, 250, or 500 $\mu$M resveratrol for 6, 24, or 48 h. After the removal of the drug, the cells were incubated for a total of 5 days after cell seeding. The cell proliferation and viability were then determined by an MTT-based colorimetric assay. The percent survival is defined as the absorbance at a given drug concentration divided by the absorbance for controls treated with DMSO alone, multiplied by 100. Each point is the average of three independent trials (nine determinations for each concentration) and presented as means: bars, SD. One-way ANOVA by Scheffe’s multiple comparison was used for statistical analyses of the extent of glioma cell cytotoxicity induced by various regimens. The significance was accepted as $P < 0.05$. 

Reverse Transcription-PCR (RT-PCR) for the Analyses of the Expression of the Vascular Endothelial Growth Factor (VEGF) in the RT-2 Glioma Cells Treated with Resveratrol. After treatment with 0, 0.1, 1, 10, 25, or 100 $\mu$M resveratrol for 24 h, the expression VEGF of the RT-2 glioma cells was studied by RT-PCR. The RNA of the cells was extracted using REzol C&T (Promega, Madison, WI) according to the manufacturer’s instructions. Semi-quantitative RT-PCR was used to assess VEGF mRNA amounts in the cells treated with resveratrol, and the expression of VEGF mRNA was determined relative to that of $\beta$-actin (housekeeping gene used as an internal standard) mRNA. Amplified products were obtained in the exponential phase for both sets of primers at 35 cycles. The differences in the expression of the RT-PCR products were analyzed by ABC-Tiger Gel V2.0 (software from Taigen Bio-science Corp., Taipei, Taiwan). The sense and antisense primers of VEGF were 5'-ATGAACCTTTCTGCTCTTGGG-3' and 5'-TCACCGCCTTGCTTGACA-3'. The sense and antisense primers of $\beta$-actin (control) were 5'-ATGGATGAC- GATATCGCTGCG-3' and 5'-GAAGGTCTCAACATGAT CTGG-3'.

The Effects of Resveratrol on the Proliferation of ECV304 HUVECs (in Vitro Proliferation Assay). The in vitro proliferation assay was used to analyze the anti-angiogenic effect of resveratrol. In triplicate wells of a flat-bottomed 12-well microtiter plate, $1 \times 10^5$ ECV304 HUVECs (prepared as mentioned above) were seeded and cultured overnight before resveratrol treatment. The ECV304 cells were exposed to 0, 0.1, 1, 5, 10, 25, 100, 250, or 500 $\mu$M resveratrol for 6, 24, or 48 h. Then MTT-based colorimetric assay (42) was used to measure the cell viability.

Immunohistochemical Studies of the s.c. Gliomas Treated with Resveratrol. The animals with s.c. tumors received various treatments immediately after tumor cell inoculation, and the tumors that were harvested at 2, 3, or 4 weeks after tumor cell inoculation (three rats at each time point) were subjected to immunohistochemical analyses. The tumors were embedded in AMES ornithine carbamyl transferase embedding compound (Miles, Elkhart, IN) and frozen at $-70^\circ$C. For immunohistochemical staining, 8-$\mu$m cryostat sections of the tumors were air-dried for 1 h at room temperature. Sections were fixed in acetone at 4°C for 5 min and washed with PBS, then incubated with 3% H$_2$O$_2$ in methanol for 30 min. The sections were then dried and incubated with blocking solution for 30 min. Next, the specific antibody was diluted in 1% BSA in PBS to optimal concentration as suggested. Mouse anti-rat CD31 antibody (PharMingen, San Diego, CA) was used in this study to monitor endothelial cell changes in CD31 expression. Another nonspecific monoclonal antibody was used as negative control. The antibodies were layered onto the section and incubated at 4°C for more than 12 h. After reacting with a secondary antibody, the sections were processed with DAKO LSAB®2 System horseradish peroxidase (DAKO Corp., Carpinteria, CA).

Fig. 2 Apoptosis of the RT-2 glioma cells subsequent to resveratrol treatment. After treatment with 0, 0.1, 1, 5, 10, 25, or 100 $\mu$M resveratrol for 6, 24, or 48 h, the apoptosis fraction of the RT-2 glioma cells was analyzed using a FACS. Each point is the average of three independent trials (nine determinations for each concentration) and presented as means: bars, SD. One-way ANOVA by Scheffe’s multiple comparison was used for statistical analyses of glioma cell apoptosis induced by various regimens. The significance was accepted as $P < 0.05$. 

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The procedure was performed according to the manufacturer’s instructions. The slides were then counterstained with hematoxylin, mounted, coverslipped, and viewed under a light microscope. The number of CD31-stained sites in the gliomas was counted, and these counts represented the microvessel density (46). Briefly, low power light microscopy (magnification ×40 and ×100) was used to scan the often heterogeneous tumor sections for areas of highest neovascularization. Any single positive-stained cell or cluster of endothelial cells that was clearly separate from adjacent microvessels, tumor cells, and other connective tissue elements was considered to be a vessel. Neither the presence of RBCs nor a vessel lumen was required for a structure to be classified as a microvessel. Individual microvessels were counted in the three areas of highest vascular density on a 200× field (×20 objective and ×10 ocular). The microvessel density (MVD) was expressed as the mean number of vessels in these areas.

**Terminal Deoxynucleotidyl Transferase-mediated dUTP Nick-End Label (TUNEL) Staining of s.c. Gliomas Treated with Resveratrol.** The animals with s.c. tumors received various treatments immediately after tumor cell inoculation, and the tumors that were harvested at 2, 3, or 4 weeks after tumor cell inoculation (three rats at each time point) were freshly frozen at −70°C, with TUNEL staining of the specimen then performed. For the TUNEL staining, 10-μm sections of tumors were stained using the TdT-FragEL DNA fragmentation detection kit (Oncogene, Boston, MA), with all procedures performed according to the manufacturer’s instructions. The sections were then counterstained with methyl green. The number of positively TUNEL-stained cells was counted under a light microscope (×200), and the apoptotic cell number (ACN) in each section was determined by averaging the cell numbers from five independent fields. The difference in the number of the apoptotic cells was compared among various groups using Student’s t test for statistical analysis.

**Western Blot Analysis.** The RT-2 cells were treated with 25 or 50 μM resveratrol for 0, 15 min, 30 min, 1, 3, 6, and 24 h, and then Western blot analysis was done. The cells were lysed in a buffer containing 20 mM HEPES at pH 7.6, 75 mM NaCl, 2.5 mM MgCl₂, 0.1 mM EDTA, 0.1% Triton X-100, 0.1% SDS, 50 mM NaF, 0.5 μg/ml leupeptin, 1 μg/ml apro tin, and 100 μg/ml 4-(2-aminoethyl) benzenesulfonyl fluoride. The cell lysate was rotated at 4°C for 30 min and then centrifuged at 10,000 rpm for 10 min, and the precipitates were discarded. The concentration of protein in the supernatant was determined using a BCA protein assay kit (Pierce, Rockford, IL), with BSA being used as a reference standard. Western blot analysis was conducted by a method described previously (47). Briefly, cellular protein (20–50 μg) was loaded onto 10% SDS-polyacrylamide gels. The protein bands were then transferred electrophoretically to polyvinylidine fluoride membranes (Micron Separations Inc., Westborough, MA). Membranes were probed with anti-α-tubulin, anti- c-Jun N-terminal kinase (JNK) 1, anti-phospho-specific JNK1, anti- extracellular signal-regulated kinase (ERK) 1/2, anti-phospho-specific ERK 1/2, anti-ATF-2, or anti-phospho-specific ATF-2 (Santa Cruz Biotechnology, CA), followed by a horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology). The detection of the antibody reactions was performed with Western blotting reagent ECL (Santa Cruz Biotechnology), the resultant chemiluminescence being demonstrated as a result of exposure of Kodak Medical X-ray film (Eastman Kodak Company, Rochester, NY).
to the filter. Differences in the expression of proteins were analyzed by ABC-Tiger Gel V2.0 (software from Taigen Bioscience Corp., Taipei, Taiwan).

**Statistical Analyses.** One-way ANOVA by Scheffe’s multiple comparison was used for statistical analyses of the extent of glioma cell cytotoxicity and apoptosis, the proliferation of the ECV304 HUVECs induced by various concentrations and drug exposure time of resveratrol, and the difference in the tumor size among various groups. Fisher’s exact test was used to analyze the animal survival rates, the Kaplan-Meier method was used to assess the animal survival time, and the log-rank statistic was used to test differences between groups. The differences in microvessel density among various groups at different time points after tumor cell inoculation were analyzed by the Mann-Whitney U test. Ps of <0.05 were considered to be statistically significant.

**RESULTS**

**Cytotoxicity Effects of Resveratrol on the RT-2 Glioma Cells.** To comprehend the inhibitory effects of resveratrol on the RT-2 glioma cells, a cytotoxicity assay was conducted as described above. Fig. 1 reveals the survival curve of the glioma cells treated with various concentrations of resveratrol and for various exposure times. The higher the resveratrol dosage and the longer the drug exposure time, the greater the proportion of cells that were killed ($P < 0.05$). The survival curves were shifted to the left when longer drug exposure times were used. The IC$_{50}$ was 164.7, 46.8, and 12.8 µM after 6, 24, and 48 h of resveratrol treatment, respectively. These data indicate that resveratrol exerts a significant cytotoxic effect upon the glioma cells in a concentration- and time-dependent manner.

**Induction of Apoptosis of the Glioma Cells by Resveratrol.** The apoptosis of the glioma cells treated with resveratrol was analyzed by FACScan, and the fraction of apoptotic glioma cells was found to be directly related to both the selected resveratrol concentration and the drug exposure time (Fig. 2). The higher the resveratrol dosage and the longer the drug exposure time, the more cells that became apoptotic ($P < 0.05$). In glioma cells treated with DMSO for 6, 24, or 48 h (controls), 3.8 to 5.6% (mean) showed apoptosis; by contrast, treatment with 25 µM resveratrol for 6, 24, or 48 h induced apoptosis in 5.9, 19.1, and 39.2% (mean) of cells, respectively, and treatment with 100 µM (the highest dosage we tested in this study) for 6, 24, or 48 h induced apoptosis in 16.9, 41.5, and 59.1% (mean) of cells, respectively. The results suggested that resveratrol induced cellular apoptosis of the glioma cells in a concentration- and time-dependent manner.

**Antitumor Effects of Resveratrol on s.c. Gliomas.** The rats were inoculated subcutaneously with $10^5$ RT-2 cells over the right flank, followed by various treatments starting immediately (small s.c. glioma model) or at day 5 (large s.c. glioma model) after tumor cell inoculation. During the 4-week treatment period, the animals treated with resveratrol or propylene glycol showed good activity with normal food and water intake and had no body weight loss, which indicated the rats tolerated the treatment well. However, the animals had body weight loss and decreased appetite when the tumor became large.

For the small s.c. gliomas (Fig. 3A), all of the rats in the groups A (no treatment), B (treated with propylene glycol), and C (treated with 10 mg/kg/day of resveratrol) died with the survival time of 48.4 ± 10.5 (mean ± SD), 50.1 ± 17.6, and 45.4 ± 12.2 days, respectively. The survival time of the rats in the groups A, B, and C were not significantly different ($P > 0.05$). The survival curves and tumor growth rates of rats with large s.c. gliomas treated with resveratrol. The rats (10/group) were s.c. inoculated with $10^5$ RT-2 cells over the right flank, followed by no treatment (group A-1), i.p. injection of propylene glycol (group B-1; vehicle; 0.5 ml), or 40 mg/kg/day of resveratrol (in 0.5 ml of propylene glycol; group D-1) for 4 weeks, starting at day 5 after tumor cell inoculation. A, the survival curves. Survival was monitored over a period of 150 days. The Kaplan-Meier method was used to assess the survival of the rats in these four groups, and the log-rank statistic was used to test for differences between groups. The survival times of the groups A-1 and B-1 were not different ($P = 0.87$). In contrast, the survival time of the group D-1 rats was significantly longer than that of the rats of the other two groups ($P = 0.00001$), with 30% of the rats having long-term survival and the survival time of the dead rats being >60 days after tumor inoculation. The survival rate among the groups A-1, B-1, and D-1 rats was not different ($P = 0.11$). B, growth rates. The tumor size was measured twice a week. The volume of the tumor was calculated from the formula $V = \frac{1}{2} (d_1 \times d_2 \times d_3)$, where $d_1$, $d_2$, and $d_3$ were mutually perpendicular diameters measured by calipers. Each point represents the average volume (cm$^3$) of the tumors for each of these groups. Daily average tumor volumes for each group were compared throughout the course of the experiment using ANOVA and the post hoc Scheffe’s multiple comparison. Group averages were not compared after one or more animals in the group died. The tumor growth rates did not differ in the groups A-1 and B-1 rats ($P > 0.05$). In contrast, the tumor size in the group D-1 rats was significantly smaller than that in the groups A-1 and B-1 rats from days 17 to 31 ($P < 0.05$).
In contrast, the animal survival time in the group D (treated with 40 mg/kg/day of resveratrol) was significantly longer than in the other three groups ($P < 0.00001$), with 70% of the rats having long-term survival and the survival time of the dead rats being more than 85 days after tumor cell inoculation. The survival rate of the rats in the group D was significantly higher than that in groups A, B, and C ($P = 0.005$). The results indicated that treatment with 40 mg/kg/day of resveratrol exerted antitumor effects on the small s.c. gliomas (i.e., increased survival rate and prolonged survival time), whereas low-dose resveratrol did not.

For the large s.c. gliomas (Fig. 4A), all of the rats in the groups A-1 (no treatment) and B-1 (treated with propylene glycol) died, with the survival time of 46.3 ± 10.9 and 47.9 ± 9.6, respectively. The survival time of the rats in groups A-1 and B-1 were not significantly different ($P = 0.87$). In contrast, the animal survival time in the group D-1 (treated with 40 mg/kg/day of resveratrol) was significantly longer than in the other two groups ($P = 0.00001$), with the survival time of the dead rats being more than 60 days after tumor cell inoculation. However, the survival rate of the rats among the groups A-1, B-1, and D-1 was not different (0% versus 0% versus 30%; $P = 0.11$). The results indicated that treatment with 40 mg/kg/day of resveratrol exerted antitumor effects on the large s.c. gliomas, as shown by prolonging the animal survival time, without affecting the animal survival rate. The animal survival of the small and large s.c. gliomas treated with 40 mg/kg/day was further compared, and we found that resveratrol was more effective for the small s.c. gliomas than the large ones, as shown by the longer animal survival time in the former ($P = 0.03$), but there was no difference of the animal survival rate between the small and large glioma groups ($P = 0.09$).

Fig. 3B shows the growth rate of the small s.c. gliomas receiving various treatments. The tumor growth rates in the groups A, B, and C did not differ significantly ($P > 0.05$). In contrast, mean tumor size in the group treated with 40 mg/kg/day of resveratrol (group D) was significantly smaller than that in group A at days 17, 25, and 28 ($P < 0.05$), smaller than that in group B at day 28 ($P < 0.05$), and smaller than that in group C at days 21 and 25 ($P < 0.05$). Fig. 4B shows the growth rate of the large s.c. gliomas receiving various treatments. The tumor growth rates in the groups A-1 and B-1 did not differ significantly ($P > 0.05$). In contrast, mean tumor size in the group treated with 40 mg/kg/day (group D-1) was significantly smaller than that in the groups A-1 and B-1 from days 17 to 31 ($P < 0.05$). The results indicated that resveratrol (40 mg/kg/day) could suppress the growth of both small and large s.c. gliomas.

**Antitumor Effects of Resveratrol on the Intracerebral Gliomas.** The rats were intracerebrally inoculated with $5 \times 10^5$ RT-2 cells, followed by various treatments starting immediately (small intracerebral glioma model) or at day 3 after tumor cell inoculation (large intracerebral glioma model). The treatment was continued until a 4-week treatment was completed or the animal died, whichever came first.

For the small intracerebral gliomas (Fig. 5A), all of the rats in the groups E (no treatment), F (treated with propylene glycol), G (treated with 10 mg/kg/day of resveratrol), and H (treated with 40 mg/kg/day of resveratrol) died and had similar survival times of 15.1 ± 2.5, 16.2 ± 2.7, 16.6 ± 2.0, and 17.7 ± 3.6 days, respectively ($P > 0.05$). In contrast, the survival time of the dead group I rats (treated with 100 mg/kg/day of resveratrol; $22.6 \pm 5.4$ days) was significantly longer than that of the other four groups ($P < 0.02$), with 1 of the 10 rats having long-term survival. The results indicated that treatment with 100 $mg/kg/day$ of resveratrol significantly prolonged the animal survival time, without affecting the animal survival rate. The animal survival of the large and small s.c. gliomas treated with 40 mg/kg/day was further compared, and we found that resveratrol was more effective for the small s.c. gliomas than the large ones, as shown by the longer animal survival time in the former ($P = 0.03$), but there was no difference of the animal survival rate between the small and large glioma groups ($P = 0.09$).

**Fig. 5** The survival curves of the rats with intracerebral gliomas treated with resveratrol. The rats (10/group) were intracerebrally inoculated with $5 \times 10^5$ RT-2 cells, followed by various treatments starting immediately (small intracerebral glioma model) or at day 3 after tumor cell inoculation (large intracerebral glioma model). The treatment was continued until a 4-week treatment was completed or the animal died, whichever came first. A, the survival curves of the rats with small intracerebral gliomas. Group E received no treatment. Groups F, G, H, and I were treated with i.p. injection of propylene glycol (vehicle, 0.5 ml), 40 mg/kg/day of resveratrol (in 0.5 ml of propylene glycol), 10 mg/kg/day of resveratrol (in 0.5 ml of propylene glycol), or 100 mg/kg/day of resveratrol (in 0.5 ml of propylene glycol), once daily, respectively. B, the survival curves of the rats with large intracerebral gliomas. Group E-1 received no treatment. Groups F-1 and I-1 were treated with i.p. injection of propylene glycol (vehicle, 0.5 ml) or 100 mg/kg/day of resveratrol (in 0.5 ml of propylene glycol), once daily, respectively. Then the animal survival time and survival rate were followed. Animals surviving >100 days were considered to be long-term survivors. Survival rates and survival times were compared among these groups. The Kaplan-Meier method was used to assess the survival of the rats in these groups, and the log-rank statistic was used to test for differences between groups. The survival rates of the rats were analyzed by Fisher’s exact test.
mg/kg/day of resveratrol exerted antitumor effects on the small intracerebral gliomas and prolonged the animal survival, whereas low-dose resveratrol did not.

For the large intracerebral gliomas (Fig. 5B), all of the rats in the groups E-1 (no treatment), F-1 (treated with propylene glycol), and I-1 (treated with 100 mg/kg/day of resveratrol) died. The groups E-1 and F-1 had similar survival times of 16.6 ± 2.2 (± SD) and 17.0 ± 2.3 days, respectively (P = 0.36). In contrast, the survival time of the group I-1 rats (20.9 ± 4.1 days) was significantly longer than that of the other two groups (P < 0.02). The results indicated that treatment with 100 mg/kg/day of resveratrol exerted antitumor effects on the large intracerebral gliomas and prolonged animal survival.

The animal survival of the small and large intracerebral gliomas treated with 100 mg/kg/day was further compared, and we found there was no difference of the animal survival time and survival rate between these two groups (P = 0.15). The results indicated that 100 mg/kg/day of resveratrol was effective for both small and large intracerebral gliomas, although resveratrol seemed to be more effective for the small than the large intracerebral gliomas because the animal survival time of the small glioma group was slightly longer than the large glioma group.

Resveratrol Suppressed the Expression of VEGF in RT-2 Glioma Cells. The expression of VEGF in RT-2 glioma cells (treated with various concentrations of resveratrol for 24 h) was studied by RT-PCR (Fig. 6A). VEGF expression did not significantly change when the glioma cells were treated with low-dose resveratrol, but it was suppressed when they were treated with 10, 25, or 100 μM resveratrol (0.7-, 0.5-, and 0.2-fold of the control, respectively). The data indicated that resveratrol suppressed the expression of VEGF in glioma cells in a concentration-dependent manner.

Inhibition of the Proliferation of the ECV304 HUVECs by Resveratrol. The in vitro proliferation assay was used to analyze the effect of resveratrol on the endothelial cells (Fig. 6B). The proliferation of ECV304 HUVECs was suppressed by resveratrol in a concentration- and time-dependent manner. Higher doses of resveratrol significantly suppressed the proliferation of ECV304 cells than lower doses (P < 0.05). The IC50 was 240.1, 70.0, and 54.5 μM after 6, 24, and 48 h of resveratrol treatment, respectively. The results indicated that resveratrol could inhibit the proliferation of ECV304 human umbilical vein endothelial cells in vitro.

Decreased Microvessel Density in s.c. Gliomas Treated with Resveratrol. The MVD of s.c. gliomas treated or not treated with resveratrol is shown in Table 1. At 14 days after tumor cell inoculation, no significant difference was found in the mean MVD (35.0, 32.2, 35.7, and 33.7; P > 0.1, Mann-Whitney U test) of group A (no treatment), B (treated with propylene glycol), C (10 mg/kg/day of resveratrol), and D (40 mg/kg/day of resveratrol) gliomas, respectively. Higher doses of resveratrol significantly suppressed the MVDs of small and large s.c. gliomas (mean MVD, 31.8 versus 19.9, P < 0.05). The ACNs and Fig. 9 shows the TUNEL staining in resveratrol-treated s.c. gliomas. For group A (no treatment)
gliomas, the mean ACN was 19.1 ± 3.6 (± SD) at week 2, 26.8 ± 4.0 at week 3, and 32.7 ± 5.7 at week 4. The ACNs for weeks 2 and 3 and for weeks 3 and 4 were not significantly different ($P > 0.05$), but the ACN at week 4 was higher than the ACN at week 2 ($P < 0.05$). For group B (treated with propylene glycol) gliomas, the mean ACN was 19.9 ± 4.0 at week 2, 24.8 ± 3.6 at week 3, and 31.3 ± 2.8 at week 4. The ACNs for weeks 2 and 3 and for weeks 3 and 4 were not significantly different ($P > 0.05$), but the ACN at week 4 was higher than the ACN at week 2 ($P < 0.05$). For group C (treated with 10 mg/kg/day of resveratrol) gliomas, the ACN was 32.9 ± 5.3 at week 2, 48.4 ± 6.9 at week 3, and 57.4 ± 6.0 at week 4, with the ACN being higher at week 3 or 4 than at week 2 ($P < 0.05$). However, no difference was found between the ACNs at weeks 2 and 3 ($P > 0.1$). For group D (treated with 40 mg/kg/day of resveratrol) gliomas, the mean ACN was 98.7 ± 15.5 at week 2, 103.7 ± 7.4 at week 3, and 176.2 ± 19.0 at week 4, with the ACN being higher at week 4 than the ACN at week 2 or 3 ($P < 0.02$). However, no difference was found between the ACNs at weeks 2 and 3 ($P > 0.1$). The data suggested that the ACN increased gradually from week 2 to week 4 in each group. Besides, the ACN of groups A and B showed no difference at each time point ($P > 0.1$). In contrast, the ACNs of groups C and D were significantly higher than those of groups A or B at all time points studied ($P < 0.05$ for group C versus group A or B; $P < 0.01$ for group D versus group A or B). In addition,

Table 1  Microvessel density of the subcutaneous gliomas treated with resveratrol

<table>
<thead>
<tr>
<th>Treatment</th>
<th>14 days$^a$</th>
<th>21 days</th>
<th>28 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A (No treatment)</td>
<td>35.0 ± 12.0 (21–56)$^b$</td>
<td>46.4 ± 9.1 (35–61)</td>
<td>44.4 ± 8.2 (30–59)</td>
</tr>
<tr>
<td>Group B (Propylene glycol)</td>
<td>32.2 ± 10.6 (19–53)</td>
<td>47.7 ± 12.3 (35–69)</td>
<td>44.3 ± 7.2 (32–56)</td>
</tr>
<tr>
<td>Group C (10 mg/kg/day Res$^c$)</td>
<td>35.7 ± 14.7 (1–63)</td>
<td>49.8 ± 19.2 (21–86)</td>
<td>31.8 ± 12.7 (15–53)</td>
</tr>
<tr>
<td>Group D (40 mg/kg Res)</td>
<td>33.7 ± 9.1 (21–51)</td>
<td>24.4 ± 8.5 (10–34)$^{d,e}$</td>
<td>19.9 ± 8.9 (7–35)$^d$</td>
</tr>
</tbody>
</table>

$^a$ Days after tumor cell inoculation.
$^b$ Individual microvessels were counted in the three areas of highest vascular density in a ×200 field (×20 objective and ×10 ocular). The microvessel density was expressed as the mean number of vessels in these areas [mean ± SD (range)]. The difference in the microvessel density among various groups at different time points after tumor cell inoculation was analyzed by the Mann-Whitney $U$ test.
$^c$ Resveratrol.
$^d$ The microvessel density in group D gliomas was significantly lower than that in group A and B gliomas ($P < 0.002$, Mann-Whitney $U$ test).
$^e$ The microvessel density in group D gliomas was significantly lower than that in group C gliomas ($P < 0.01$, Mann-Whitney $U$ test).

Fig. 7 Immunohistochemical staining for microvessels in glioma tissues. Representative photomicrographs of sections of glioma (at day 28 after tumor cell inoculation) immunohistochemically stained for CD31 are shown. Rats received either no treatment (group A), propylene glycol (group B), 10 mg/kg/day of resveratrol (group C), or 40 mg/kg/day of resveratrol (group D). The positive staining represents the microvessels (×100).

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Effects of Resveratrol on Gliomas

The observed resveratrol-induced apoptosis of the RT-2 glioma cells appeared to occur in a concentration- and time-dependent manner, with higher concentrations and prolonged exposure eliciting significant cellular apoptosis. Our resveratrol-induced cytotoxicity and apoptosis data suggest that prolonged treatment with resveratrol at the micromolar serum level might be a possible treatment strategy for gliomas. In addition, our results demonstrated that treatment with certain resveratrol concentrations cause more glioma cell cytotoxicity than apoptosis, suggesting that other mechanisms, independent of the apoptosis process, may contribute to the cytotoxic effects of resveratrol in glioma cells.

Because resveratrol caused cytotoxic effects and apoptosis in the glioma cells, we further investigated the in vivo effects of resveratrol on the gliomas. We found that high-dose (40 mg/kg/day) resveratrol [unlike no or low-dose resveratrol (10 mg/kg/day)] slowed the growth of s.c. tumors, prolonged animal survival time, and increased animal survival rate. Furthermore, we found that resveratrol was more effective for the small s.c. gliomas than the large ones (resveratrol treatment was started at day 5 after tumor cell inoculation). The less antitumor effect on large tumors was a common problem seen in many other treatment strategies for the malignant tumors in clinical situation. The in vivo antitumor effects of resveratrol had been studied in several reports (28, 48). One report found that i.p. injection of 2.5 or 10 mg/kg/day of resveratrol for 3 weeks effectively prevented s.c. tumor growth and metastasis to lung in Lewis lung carcinoma-bearing mice (28). Another report demonstrated that oral administration of 1 mg/kg/day of resveratrol suppressed the growth of s.c. fibrosarcomas in mice (48). These two reports revealed that low doses of resveratrol had antitumor effects. By contrast, our study revealed that only a high dose (40 mg/kg/day) had antitumor effects, whereas 10 mg/kg/day (similar to or higher than the doses used in the literature) was not effective for the treatment of s.c. gliomas. Such differences might be related to the animal models used, the inoculation amount, characteristics of the tumor cells, the plasma level of resveratrol, etc. We did not measure and do not know the plasma level of resveratrol in the rats treated with 40 mg/kg/day. One report mentioned that oral administration of 28 µg of resveratrol to male rats achieves a peak plasma level >20 ng/ml after 1 h (49); accordingly, we estimated that a single dose of 40 mg/kg might result in a peak plasma level of ~25 µM. Such a plasma level was in the range of the IC₅₀ levels we found by cytotoxicity assay and was presumed to have therapeutic effect. Because the effect of resveratrol on s.c. gliomas does not represent its effect on the intracerebral gliomas, we studied the latter. We found that 40 mg/kg/day of resveratrol had no therapeutic effect on the intracerebral gliomas, but 100 mg/kg/day did prolong animal survival time in both small and large tumors. In addition, under the treatment of 100 mg/kg/day of resveratrol, the rats with large intracerebral gliomas seemed to have shorter survival time than those with large ones, although statistically the difference of the animal survival time between these two groups was not different. Furthermore, this antitumor effect on intracerebral gliomas was not as good as the effects seen on the s.c. gliomas. The smaller antitumor effect and the need for a higher dose were the reasons why we further investigated the in vivo effects of resveratrol on the gliomas. We found that high-dose (40 mg/kg/day) resveratrol [unlike no or low-dose resveratrol (10 mg/kg/day)] slowed the growth of s.c. tumors, prolonged animal survival time, and increased animal survival rate. Furthermore, we found that resveratrol was more effective for the small s.c. gliomas than the large ones (resveratrol treatment was started at day 5 after tumor cell inoculation). The less antitumor effect on large tumors was a common problem seen in many other treatment strategies for the malignant tumors in clinical situation.

**Fig. 8** The number of the apoptotic s.c. glioma cells from rats treated with or without resveratrol. TUNEL staining of the tumors was performed to elucidate the effect of resveratrol on glioma cell apoptosis. The rats received either no treatment (group A), propylene glycol (group B), 10 mg/kg/day of resveratrol (group C), or 40 mg/kg/day of resveratrol (group D) for 4 weeks. The tumors were harvested at 2, 3, and 4 weeks after tumor cell inoculation, with three rats at each time point. The number of cells positively stained by TUNEL staining was counted under microscope (×200), and the ACN in each section was determined by averaging the cell numbers from five independent fields. The ACN is presented as means; bars, SD. The difference of the number of the apoptotic cells was compared among various groups using Student’s t test for statistical analysis. Results were considered statistically significant where P < 0.05. The ACN at each time point was statistically not different between groups A and B (P > 0.1). *, the ACN of group C or D was higher than the ACN of group A or B at that time point (P < 0.05). †, the ACN of group D was higher than the ACN of group C at that time point (P < 0.01). #, the ACN at week 3 or 4 was higher than at the week 2 in the same group (P < 0.05). @, the ACN at week 4 was higher than at the week 3 in the same group (P < 0.01).

Resveratrol Caused No Significant Change in the MAPK Expression of Glioma Cells. Western blot analysis revealed no change in MAPKs (JNK1, ERK1/2, and p38 MAPK) and downstream protein (ATF-2, and their phospho-specific proteins from RT-2 cells treated with 25 or 50 µM resveratrol for various time periods (Fig. 10). The results suggested that resveratrol did not affect the MAPK signaling pathway in glioma cells.

**DISCUSSION**

In this study, we found that resveratrol elicited a concentration- and time-dependent inhibition of glioma cell proliferation, with the IC₅₀ in the micromolar range. This IC₅₀ was comparable to previously reported IC₅₀ for other tumors such as leukemia, prostate, breast, and colon cancers (13–17). Because defective control of apoptosis has been considered to play a central role in the pathogenesis of tumors (33), we further studied the effect of resveratrol on the induction of glioma cell apoptosis and noted that resveratrol also induced apoptosis in glioma cells, as was seen in a variety of other cancer cells (13, 17, 18, 20, 27). The observed resveratrol-induced apoptosis of the RT-2 glioma cells appeared to occur in a concentration- and time-dependent manner, with higher concentrations and prolonged exposure eliciting significant cellular apoptosis. Our resveratrol-induced cytotoxicity and apoptosis data suggest that prolonged treatment with resveratrol at the micromolar serum level might be a possible treatment strategy for gliomas. In addition, our results demonstrated that treatment with certain resveratrol concentrations cause more glioma cell cytotoxicity than apoptosis, suggesting that other mechanisms, independent of the apoptosis process, may contribute to the cytotoxic effects of resveratrol in glioma cells.
These angiogenic factors, VEGF is an important angiogenic factor and essential in tumorigenesis in different types of human cancers (61–63). In addition, VEGF is a prognostic indicator of the severity of cancers such as breast cancer (62). Furthermore, resveratrol has been found to inhibit the binding of VEGF to HUVECs (28), capillary endothelial cell growth and proliferation (27, 28, 48, 51), and capillary formation by endothelial cells (28). In gliomas, angiogenesis has also been found related to the amount of secreted VEGF (57, 64), and in this study, we found that resveratrol suppressed VEGF expression in glioma cells and inhibited the proliferation of the HUVECs in a concentration- and time-dependent manner. The inhibition of the proliferation of the endothelial cells might, at least indirectly and partially, represent the suppression of the vessel formation. All these data indicated that resveratrol might have an anti-angiogenesis effect on the glioma. In addition, we further demonstrated that resveratrol suppressed the glioma-induced angiogenesis in vivo as shown by the decreased MVD in the gliomas treated with a high dose (40 mg/kg/day) of resveratrol, relative to the MVDs of the control (untreated gliomas) and of gliomas treated with a low dose of resveratrol (10 mg/kg/day). Thus, resveratrol seemed to inhibit glioma-induced angiogenesis and thereby possibly contribute to the antitumor effect of resveratrol on the gliomas.

The biological effects of VEGF are mediated by two tyrosine kinase receptors, KDR (kinase domain region) and flt-1 (fms-like tyrosine kinase 1), which bind VEGF with high affinity (65). Furthermore, the FDR/flk-1 receptor is believed to mediate the mitogenic stimulus in response to VEGF, and the proliferative effects of VEGF after binding to KDR/flk-1 on endothelial cells are mediated, at least in part, by activation of the MAPK signaling pathway (66). VEGF and its receptor may have either autocrine or paracrine effects on the tumor cells and activate the MAPK pathway, leading to tumor formation, invasion, and production of angiogenic factors (62, 65, 67). MAPK consists at least of four subtypes, including ERK, p38 MAPK, ERK5/big MAPK 1 (BMK1), and JNK (68). In different cell lines, these MAPKs have been shown to play an important role...
in the regulation of apoptosis in response to different stimuli (20, 35–39). VEGF has been found to induce the activation of the ERK1/2 pathway in breast cancer cells and human pancreatic cells (62, 65); by contrast, the activation of VEGF secretion and angiogenic response in breast cancer cells has been found to be induced through the activation of p38 MAPK (69). Another report mentioned, however, VEGF is regulated (in a ras-transformed rat liver epithelial cell line and human breast cancer cell lines) in a positive manner by the ERK and negative manner by the p38 signaling pathways; thus, ERK and p38 seemed to play a differential role in the activation of the VEGF (34). As to the glioma cells, the ERK1/2 pathway is involved in the up-regulation of VEGF expression in human glioblastoma cells by ionizing radiation (70). As a whole, the role of various subtypes of the MAPK signaling pathway in the activation of VEGF is unclear in the literature, and further study is needed to elucidate it. In addition to finding that resveratrol induces apoptosis in glioma cells, we found that resveratrol suppressed the expression of VEGF in the glioma cells, and thus resveratrol might change the expression of the MAPK enzyme. However, the effects of resveratrol on the MAPK signaling pathway are inconsistent in the literature (19, 20, 71). Resveratrol has been demonstrated to induce apoptosis and antitumor effects through ERKs, p38 kinase, and JNK-mediated pathways in an epidermal cell line (20, 71) or ERK in human neuroblastoma cells (19). Resveratrol stimulates ERK1/2 activity in neuroblastoma cells in a wide range of concentrations (1 pm to 10 μM); however, higher concentrations (50–100 μM) inhibited MAPK phosphorylation (19). Other studies found p38 MAPK, ERKs, and/or JNK mediates resveratrol (10–40 μM)-induced apoptosis of the mouse epidermal cells, and such apoptosis is related to the activation of p53 (20, 71). In this study, although resveratrol doses and drug exposure times in our study were similar to those used in other reports (19, 20, 71), resveratrol did not cause any significant change in the expression of MAPKs. Thus, the MAPK signaling pathway seemed not related to the resveratrol-induced suppression of the glioma cell VEGF expression or apoptosis, and further studies are necessary to identify the signaling pathways that resveratrol acts through.

In summary, this study demonstrated that resveratrol caused concentration- and time-dependent cytotoxicity and induction of glioma cell apoptosis. In addition, resveratrol exerted an antitumor effect on both s.c. and intracerebral gliomas, as shown by the slower tumor growth rate and prolonged animal survival time, although the dosage of resveratrol required for effective antitumor effects was higher for the intracerebral than for the s.c. gliomas. The mechanisms of such antitumor effects of resveratrol were found to be related at least partly to the inhibition of the glioma-induced angiogenesis. To the best of our knowledge, this is the first report demonstrating the effects of resveratrol on the tumor growth and angiogenesis of gliomas. However, clinical studies are needed before making any recommendation about the use of resveratrol in the treatment of gliomas.

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Resveratrol Suppresses the Angiogenesis and Tumor Growth of Gliomas in Rats
Sheng-Hong Tseng, Swei-Ming Lin, Jin-Cherng Chen, et al.


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