# Resveratrol Suppresses Angiogenesis in Gliomas: Evaluation by Color Doppler Ultrasound

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**Abstract.** *Background: The effects of resveratrol (a natural polyphenol) on angiogenesis in rat gliomas were investigated by color Doppler ultrasound (CDUS). The correlation among the tumor growth rate, macroscopic angiogenesis measured by CDUS and microscopic angiogenesis was assessed by immunohistochemical staining. Materials and Methods: Fischer rats were subcutaneously inoculated with rat RT-2 glioma cells and treated with resveratrol for 4 weeks. The tumor size was measured and the animal survival followed. CDUS examination was used to measure tumor blood flow shown as the color Doppler vascularity index (CDVI). Immunohistochemical staining of CD31 was carried out to assess the microvessel density (MVD) of the tumors. The CDVI, MVD and tumor size were correlated. Results: Rats treated with resveratrol (40 mg/kg/day) had slower tumor growth rates than those of the control groups ($p<0.05$). The CDVI, MVD and tumor size were significantly correlated (linear regression, $p<0.0001$). Conclusion: Resveratrol-suppressed glioma growth was significantly correlated with the inhibition of macroscopic and microscopic angiogenesis.*

Resveratrol (3,4,5'-trihydroxy-trans-stilbene), a natural polyphenol, is derived mainly from grapes and mulberry (1). It has been found to have a variety of effects, such as an antioxidant effect, reduction of the synthesis of lipid in the liver, inhibition of platelet aggregation, *etc.* (1-3). In recent years, resveratrol has further been demonstrated to be an antitumor and chemopreventive agent, which can inhibit the proliferation of several kinds of solid tumors, such as prostate, breast and colon cancers, neuroblastoma, as well as leukemia (4-14). In addition, resveratrol has been found to inhibit angiogenesis in tumors, shown as the inhibition of capillary endothelial cell growth and proliferation, as well capillary formation by endothelial cells (15-18). Such antitumor and anti-angiogenesis effects of resveratrol were also demonstrated in gliomas in our previous study (19).

To study tumor angiogenesis, the immunohistochemical staining of endothelial cells to determine the microvessel density (MVD) is commonly used (19-22). Although this method gives an instantaneous assessment of vascularization, it only reveals microscopic angiogenesis, which might or might not represent the features of macroscopic angiogenesis (21-23). Ultrasound, a non-invasive technology, has been used to study neovascularization in a variety of human tumors, such as soft tissue sarcoma, colon, endometrial, ovarian, cervical and rectal cancers (24-30). Ultrasound can assess macroscopic angiogenesis in tumors, which is different from the microscopic angiogenesis revealed by MVD (25, 31, 32). Therefore, the aim of this study was to investigate the effects of resveratrol on macroscopic angiogenesis in gliomas using color Doppler ultrasound (CDUS) to measure the color Doppler vascularity index (CDVI) longitudinally, which was then correlated with the tumor growth rate. In addition, tumors were subjected to CDUS examination for CDVI calculation before tumor excision for measurement of tumor size, and tissue preparation for immunohistochemical staining of CD31 to assess the MVD. The CDVI, MVD and tumor size were connected to investigate whether macroscopic or microscopic angiogenesis was correlated with tumor growth. This is, to our knowledge, the first investigation of the anti-angiogenesis effects of resveratrol on gliomas by correlating the CDVI, MVD and tumor growth rate.

## Materials and Methods

*Cell line and cell culture.* The cell line used in this study was the rat RT-2 glioma cell line, which is derived from an avian sarcoma

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virus-induced brain tumor in the Fischer 344 rat (33). The RT-2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) at 37˚C in a 5% $CO_2$ incubator.

*Animals.* The animal experiments were conducted according to the guidelines of the Laboratory Animal Center, College of Medicine, National Taiwan University, R.O.C. Fischer rats weighing 200-350 g were used for the experiments. These rats were housed, with free access to food and water, on a 12:12-h day-night cycle (lights on from 06.00 to 18.00 h) with room temperature maintained at approximately 20˚C.

*Resveratrol treatment of subcutaneous gliomas in rats.* A subcutaneous tumor was induced by injection of $3x10^4$ RT-2 glioma cells (in 10 μl phosphate-buffered solution, PBS) into the right flank of each rat, which then began various treatments as described below. The animal experiments consisted of 4 groups of 6 rats each. Group A received no treatment. Groups B, C and D were treated with intraperitoneal injection of propylene glycol (vehicle), resveratrol 10 mg/kg (in 0.5 ml propylene glycol), or 40 mg/kg (in 0.5 ml propylene glycol), once daily for 4 weeks, respectively (19). The animal survival times and rates were followed and were compared among these 4 groups. The tumor size was measured weekly by a blinded observer until death of the rat. The tumor volume was calculated from the formula V = 1/2 (d1 x d2 x d3), with d1, d2 and d3 tumor diameters measured with calipers in mutually perpendicular directions. The average daily tumor volumes from each group were compared throughout the course of the experiment.

*Sequential CDUS examination of subcutaneous gliomas and correlation of the CDVI with tumor growth rate.* The neovascularity of the subcutaneous gliomas was studied by CDUS examination using a CDUS unit (HDI 5000; Advanced Technology Laboratories, Bothell, WA, USA) in weeks 2, 3, 4, 5 and 6 after tumor cell inoculation. The tumors usually grew to about 1 cm in diameter 2 weeks after tumor cell inoculation and a tumor size greater than 1 cm is suitable for ultrasound examination. A 5-10-MHz (intraoperative scanhead) broad band linear array transducer (26 mm in width) was used (21, 25, 34). If the tumor's cross-sectional diameter was more than 2.5 cm, a 5-12-MHz broad band linear array transducer (38 mm in width) was used. The parameters with the highest sensitivity and least interference were chosen (medium wall filter, pulsed repetition frequency 1000 Hz, moderate-to-long persistence, and velocity <5 cm/sec). The focusing depths were set at 0.5 cm and 1.5 cm. Each tumor was scanned 3 times and the 3 sections with the largest color signals were adopted for analyses. For the analysis of angiogenesis, the tumor margin was contoured using a cursor and quantitation of the vascular color signals within the demarcated tumor was automatically executed by the software Encomate (Electronic Business Machine Co., Ltd., Taipei, Taiwan, R.O.C.). The ratio of the number of colored pixels within a tumor section to the total number of pixels in that specific tumor section was calculated and designated as the CDVI (25, 34). The correlation between the CDVI data and tumor growth rate was measured.

*Correlation of the CDVI, MVD and tumor size.* The experiment consisted of 4 groups with 30 rats in each group. The glioma cells were inoculated into the right flank of the animals, which were treated as described above. These rats underwent CDUS examination of their tumors 2, 3, 4, 5 and 6 weeks after tumor cell inoculation (6 rats for each time-point), as described above. The tumors were then harvested and their sizes were measured as above, after which they were preserved in AMES Ornithine carbamyl transferase embedding compound (Miles, Elkhart, IN, USA) and frozen at –70˚C for histological and immunohistochemical analyses. To determine the relationship among the tumor growth, microscopic and macroscopic angiogenesis, the correlation among the tumor size, CDVI (measured by CDUS) and MVD (measured by the microscopic quantification of microvessels using immunohistochemistry described below) was analyzed.

*Histological and immunohistochemical studies of tumors treated with resveratrol.* Tumors harvested at 2, 3, 4, 5 or 6 weeks after tumor cell inoculation were subjected to histological (hematoxylin & eosin stain, H & E stain) and immunohistochemical analyses. The tumors were embedded in AMES Ornithine carbamyl transferase embedding compound (Miles) and frozen at –70˚C. For H & E staining, 8-μm-thick cryostat sections of the brains were fixed in acetone at 20˚C for 1 min, and then washed with PBS. The sections were stained with hematoxylin at room temperature for 3.5 min, washed and then stained with eosin for several seconds. After washing, the sections were air-dried, mounted, coverslipped and viewed under a light microscope. For immunohistochemical staining, 8-μm-thick cryostat sections of 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% $H_2O_2$ in methanol for 30 min. The sections were then dried and incubated with blocking solution for 30 min. Next, specific antibody was diluted in 1% bovine serum albumin in PBS to optimal concentration. The mouse anti-rat CD31 antibody (PharMingen, San Diego, CA, USA) was used in this study to identify changes in endothelial cells. A non-specific monoclonal antibody was used as the 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 by the avidin-biotin-peroxidase method and reacted with 3,3'-diaminobenzidine tetrahydrochloride. The slides were then counterstained with hematoxylin, mounted, coverslipped and viewed under a light microscope. The expression of CD31 in gliomas was counted as the MVD (22). Briefly, low-power light microscopy (magnification 40x and 100x) was used to scan the tumor sections for areas of greatest neovascularization. Any single positively-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 red blood cells nor a vessel lumen was required for a structure to be classified as a microvessel. Individual microvessel counts were conducted in the 3 areas of greatest vascular density on a 200x field (20x objective and 10x ocular). The MVD was expressed as the mean number of vessels in these areas.

*Statistical analyses.* The rat survival rates were analyzed by Fisher's exact test. The Kaplan-Meier method was used to assess the rat survival time and the log-rank statistic was used to test for differences among different groups. The difference between the CDVI, MVD or tumor size among various groups was compared

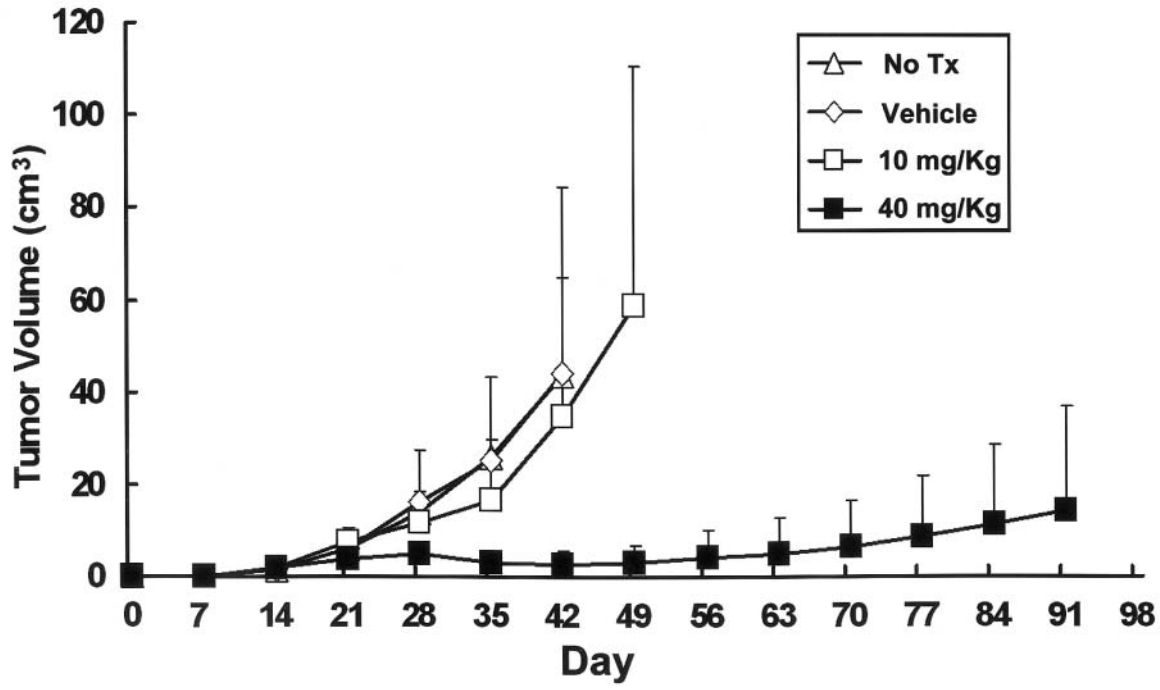


Figure 1. *The tumor growth curves of the rats treated with resveratrol. The rats (6 per group) were subcutaneously inoculated with RT-2 cells into the right flank and received no treatment (Group A), intraperitoneal injection of propylene glycol (Group B; vehicle), 10 mg/kg/day of resveratrol (Group C), or 40 mg/kg/day of resveratrol (Group D) for 4 weeks. The tumor size was measured weekly and shown as mean±standard deviation. The tumor growth rates did not differ in the Group A, B and C rats ($p>0.1$). The tumor size of Group D was not different from that of Groups A, B or C at weeks 2 and 3 ($p>0.1$); however, the former was significantly smaller than the latter at weeks 4, 5 and 6 ($p<0.05$). In addition, the tumor size of Group D was significantly less than that of Group C at week 7 ($p<0.05$).*

using the Mann-Whitney *U*-test for statistical analysis. Linear regression was used for the analyses of correlation among the tumor size, MVD and CDVI. A $p$ value of $<0.05$ was considered statistically significant.

## Results

*Antitumor effects of resveratrol on subcutaneous gliomas.* Rats were subcutaneously inoculated with RT-2 cells into the right flank and received no treatment (Group A), intraperitoneal injection of propylene glycol (Group B), 10 mg/kg/day of resveratrol (Group C), or 40 mg/kg/day of resveratrol (Group D) for 4 weeks. All rats in Groups A, B and C died, with survival times of 61.3±13.5 (mean±standard deviation [SD]), 61.5±13.4 and 72.7±16.4 days, respectively. There was no difference in survival time among Groups A, B and C ($p>0.08$). In contrast, 4 of the 6 rats in Group D had long-term survival, and the 2 rats that died did so more than 96 days after tumor cell inoculation. The rats in Group D had a significantly higher survival rate ($p=0.03$) and longer survival time ($p=0.0005$) than those in Groups A, B and C. These results indicated that treatment with 40 mg/kg/day of resveratrol exerted antitumor effects on the gliomas with increases in the animal survival rate and survival time, whereas a low dose of resveratrol did not produce a significant effect.

*Sequential CDUS examination of subcutaneous gliomas and correlation of the CDVI with tumor growth rate.* The growth rates of the subcutaneous gliomas receiving the various treatments are illustrated in Figure 1. The tumor growth rates in Groups A, B and C did not differ significantly from each other at any of the time-points studied ($p>0.16$). Although the tumor size in Group D showed no difference from that in Groups A, B or C at weeks 2 and 3 ($p>0.1$), it was significantly less in Group D at weeks 4, 5 and 6 ($p<0.05$). In addition, the tumor size in Group D was significantly less than that in Group C at week 7 ($p<0.05$). The results indicated that a high dose of resveratrol (40 mg/kg/day) could suppress the growth of the subcutaneous gliomas, while a low dose of resveratrol could not.

The neovascularity of subcutaneous gliomas was studied by CDUS examination at weeks 2, 3, 4, 5 and 6 after tumor cell inoculation. Each tumor was scanned and the 3 sections with the largest color signal were adopted for analyses. All

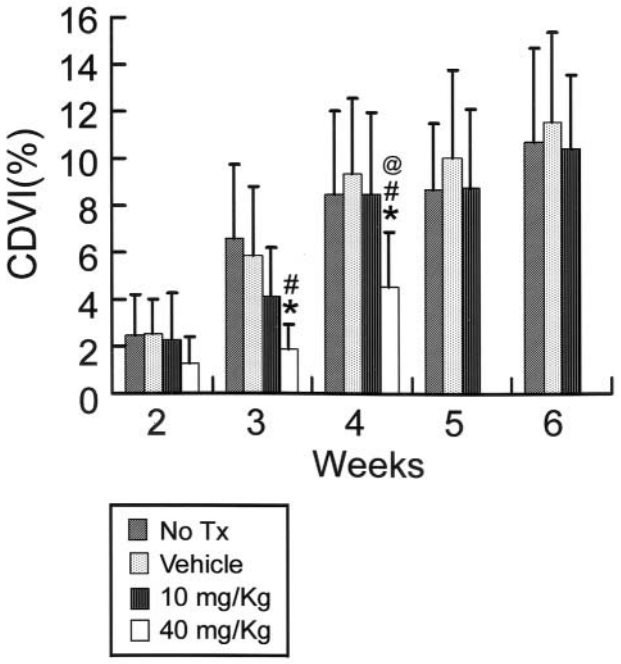


Figure 2. *The CDVI of the subcutaneous gliomas treated with resveratrol. The rats (6 per group) were subcutaneously inoculated with RT-2 cells into the right flank and received no treatment (Group A), intraperitoneal injection of propylene glycol (Group B; vehicle), 10 mg/kg/day of resveratrol (Group C), or 40 mg/kg/day of resveratrol (Group D) for 4 weeks. The neovascularity of the subcutaneous gliomas was studied by CDUS examination at weeks 2, 3, 4, 5 and 6 as described in "Materials and Methods". The result obtained was designated as the CDVI and is presented as mean±standard deviation. The CDVI at each time-point was not statistically different among Groups A, B and C ($p>0.05$). * Indicates that the CDVI of Group D was smaller than that of Group A at that time-point ($p=0.01$ at week 3; $p<0.05$ at week 4). # Indicates that the CDVI of Group D was smaller than that of Group B at that time-point ($p<0.05$ at week 3; $p<0.01$ at week 4). @ Indicates that the CDVI of Group D was smaller than that of Group C at week 4 ($p<0.05$). The CDVI was not compared between Group D and Group A, B, or C at weeks 5 and 6 because the tumor in 4 rats of Group D decreased in size at week 5 and had disappeared by week 6.*

tumors demonstrated neovascularity by CDUS, with the colored area mainly in the periphery of the tumor; the central areas were relatively anechoic. The CDVI of subcutaneous gliomas treated with various regimens are presented in Figure 2. For Groups A, B and C, the mean CDVI increased gradually from week 2 (1.27 to 2.56) to week 6 (10.37 to 11.54). The mean CDVI of Group D increased from week 2 (1.27) to week 4 (4.55). The CDVI of Group D was not calculated for weeks 5 and 6 because the tumors in 4 out of the 6 rats decreased in size at week 5 and had disappeared by week 6. The CDVI at each time-point was not statistically different among Groups A, B and C ($p>0.05$). There was also no difference in the CDVI between Group D and Groups A, B or C in week 2 ($p>0.05$). In contrast, the CDVI of Group D became significantly less than that of Groups A or B in weeks 3 and 4 ($p<0.05$). The CDVI of Group D did not differ from that of Group C in week 3 ($p=0.05$), but was significantly less in week 4 ($p<0.05$). These data suggest that the macroscopic angiogenesis of the gliomas increased along with tumor growth. The high dose of resveratrol suppressed angiogenesis during the period of treatment, but the low dose of resveratrol did not. Furthermore, a significant correlation was found between the CDVI and the tumor growth rate (linear regression, $r=0.753$, $p<0.0001$), suggesting that the suppressed macroscopic angiogenesis was correlated with the inhibited tumor growth.

*Correlation among the CDVI, MVD and tumor size.* A possible correlation between the CDVI (as measured by CDUS), MVD (by microscopic quantification of microvessels in immunohistochemical staining) and tumor size was studied. Rats in each of the 4 groups underwent CDUS examination of the tumors in weeks 2, 3, 4, 5 and 6 with measurement of each subject's CDVI. The tumors were then harvested and their sizes measured. The tumors were subjected to immunohistochemical staining of CD31 and the MVD was calculated. Because 2 tumors in Group D disappeared in week 5 and 3 tumors were smaller than 1 cm in diameter, the data for this group after week 4 could not be used for comparison. However, the tumor size could still be measured. The CDVI and MVD values, as well as the tumor size for subcutaneous gliomas receiving various treatments in weeks 2, 3, 4, 5 and 6 after tumor cell inoculation, are shown in Figure 3. There was no difference in the CDVI among Groups A, B and C from weeks 2 to 6 after tumor cell inoculation ($p>0.05$). The CDVI of Group D (treated with 40 mg/kg/day of resveratrol) did not differ from that of the other 3 groups in week 2 after tumor cell inoculation ($p>0.05$). In contrast, in weeks 3 and 4 after tumor cell inoculation, Group D had a significantly smaller CDVI than the other 3 groups (week 3; mean CDVI, 1.28 *vs.* 5.53, 5.91 or 4.38, respectively; $p<0.05$) (week 4; mean CDVI, 4.01 *vs.* 8.08, 8.66, or 7.64; $p<0.05$) (Figure 4). The data indicate that treatment with 40 mg/kg/day of resveratrol significantly suppressed macroscopic angiogenesis in the glioma, whereas 10 mg/kg/day of resveratrol did not.

The MVD value was compared among the various groups (Figure 3). There was no difference in the MVD among Groups A, B and C from week 2 to 6 after tumor cell inoculation ($p>0.1$). The MVD of Group D did not differ from the value for the other 3 groups in week 2 ($p>0.1$). In contrast, in weeks 3 and 4 after tumor cell inoculation, Group D showed a significantly smaller MVD value than the other 3 groups (week 3; mean MVD, 24.0 *vs.* 49.2, 49.0 or 50.7, respectively; $p<0.05$) (week 4; mean MVD,

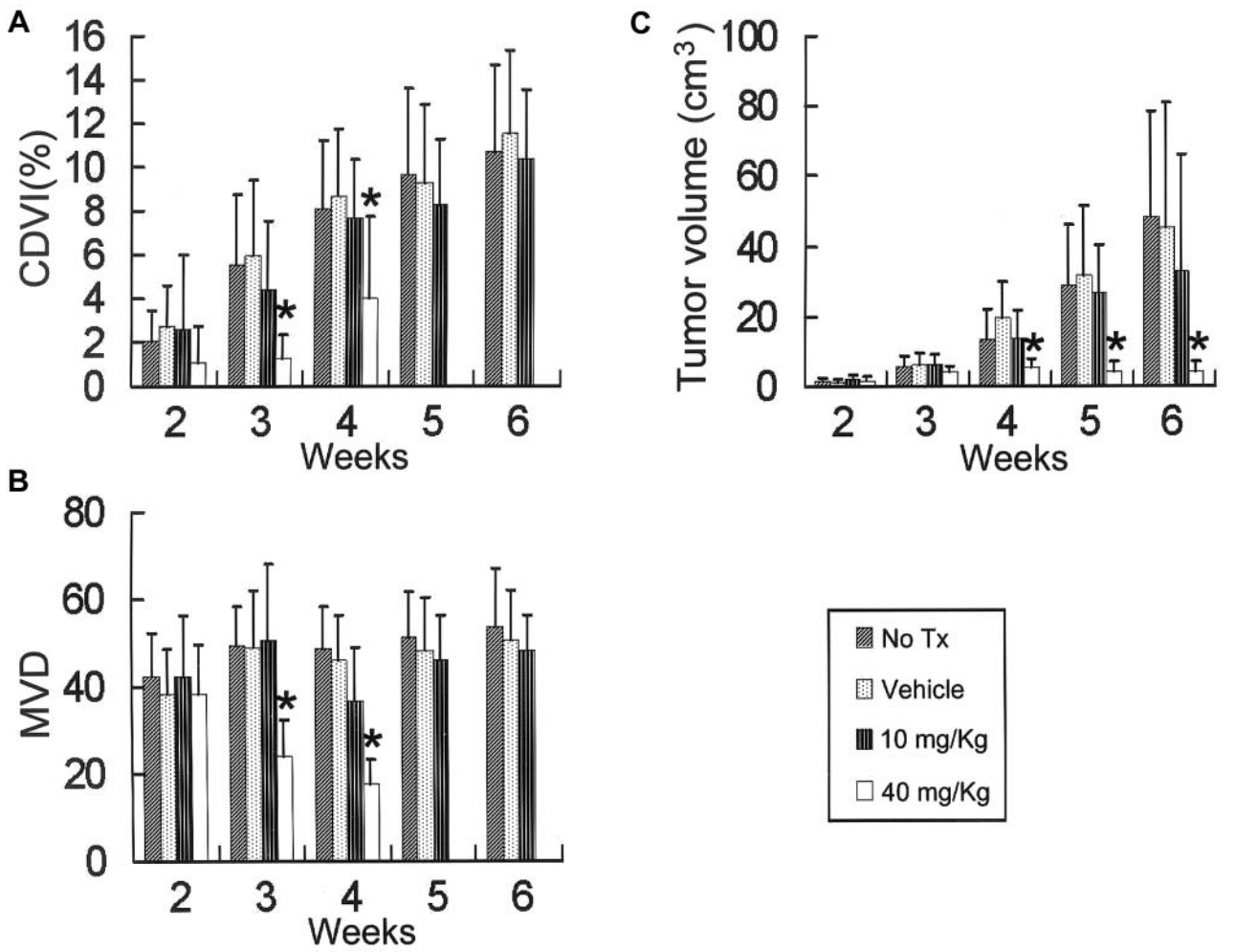


Figure 3. *Correlation of the CDVI, MVD and tumor growth rates of the subcutaneous gliomas. The rats were subcutaneously inoculated with RT-2 cells into the right flank and received no treatment (Group A), intraperitoneal injection of propylene glycol (Group B; vehicle), 10 mg/kg/day of resveratrol (Group C) or 40 mg/kg/day of resveratrol (Group D) for 4 weeks. The CDVI of the subcutaneous gliomas was measured by CDUS examination at weeks 2, 3, 4, 5 and 6, and was presented as mean±standard deviation. (A) After CDUS examination, the tumors were harvested and subjected to immunohistochemical staining of CD31 and the MVD was counted and presented as mean±standard deviation. (B) The tumor size was measured and shown as the mean±standard deviation. (C) The difference of the CDVI, MVD and tumor size was compared among the groups using the Mann-Whitney U-test for statistical analysis. Results were considered statistically significant where $p<0.05$. Because 2 tumors in Group D disappeared at week 5 and 3 tumors were smaller than 1 cm diameter, the data for this group after week 4 could not be used for comparison. * Indicates that the CDVI, MVD, and tumor size of Group D was smaller than that of Groups A, B or C at that time-point ($p<0.05$). Linear regression analysis of the correlation was determined between the CDVI and MVD ($r=0.729$, $p<0.0001$), between CDVI and tumor growth rate ($r=0.794$, $p<0.0001$) and between the MVD and tumor size ($r=0.567$, $p<0.0001$).*

17.7 *vs.* 48.7, 46.0 or 36.7, respectively; $p<0.01$) (Figure 5). The data indicated that treatment with 40 mg/kg/day of resveratrol suppressed microscopic angiogenesis in the glioma, while 10 mg/kg/day of resveratrol did not.

Tumor sizes at different time-points after inoculation were compared among the various groups (Figure 3). There was no difference in the tumor size among Groups A, B and C at any of the time-points studied ($p>0.1$). Although the tumor size in Group D showed no difference from that in the other groups in weeks 2 and 3 ($p>0.1$), it was significantly less in Group D than Groups A, B or C in weeks 4, 5 and 6 ($p<0.05$). These results indicated that a high dose of resveratrol (40 mg/kg/day) could suppress the growth of the subcutaneous gliomas, while a low dose of resveratrol could not.

The correlation among the CDVI, MVD and tumor size was further analyzed by linear regression analyses. The CDVI and MVD were statistically significantly correlated ($r=0.729$, $p<0.0001$), suggesting that the subcutaneous

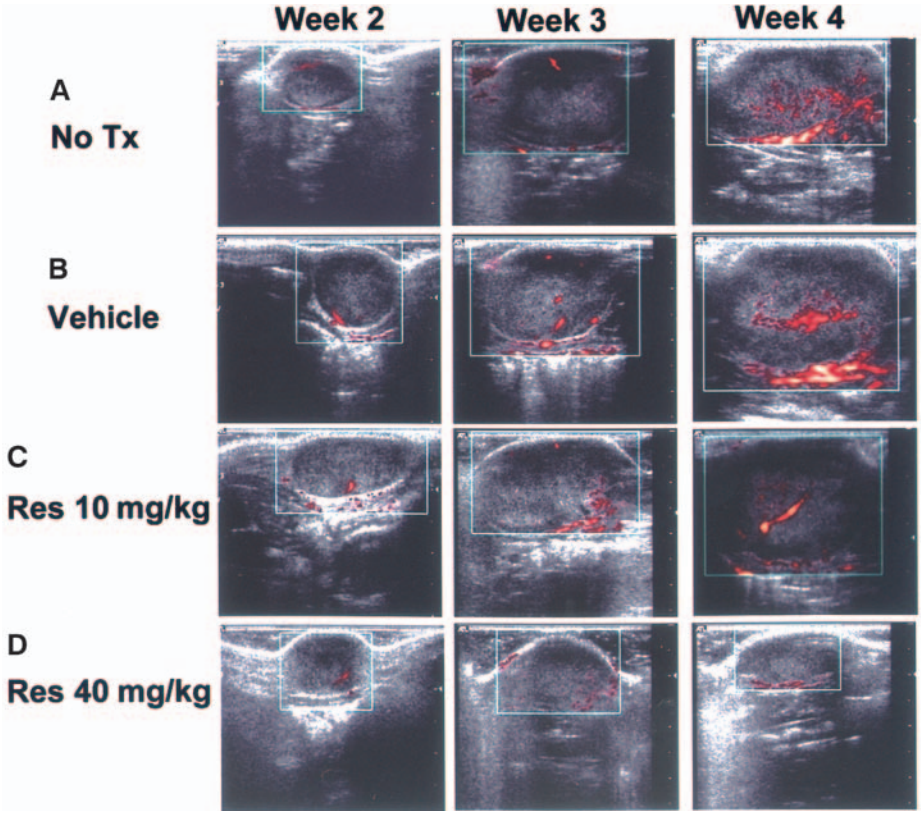


Figure 4. *Photograph of the neovascularity of the subcutaneous gliomas by CDUS examination. Representative photomicrographs of the color Doppler ultrasound of the gliomas (surrounded by a rectangle) of the rats at week 4 post tumor cell inoculation (A, no treatment; B, treatment with propylene glycol; C, treatment with 10 mg/kg/day of resveratrol; D, treatment with 40 mg/kg/day of resveratrol).*

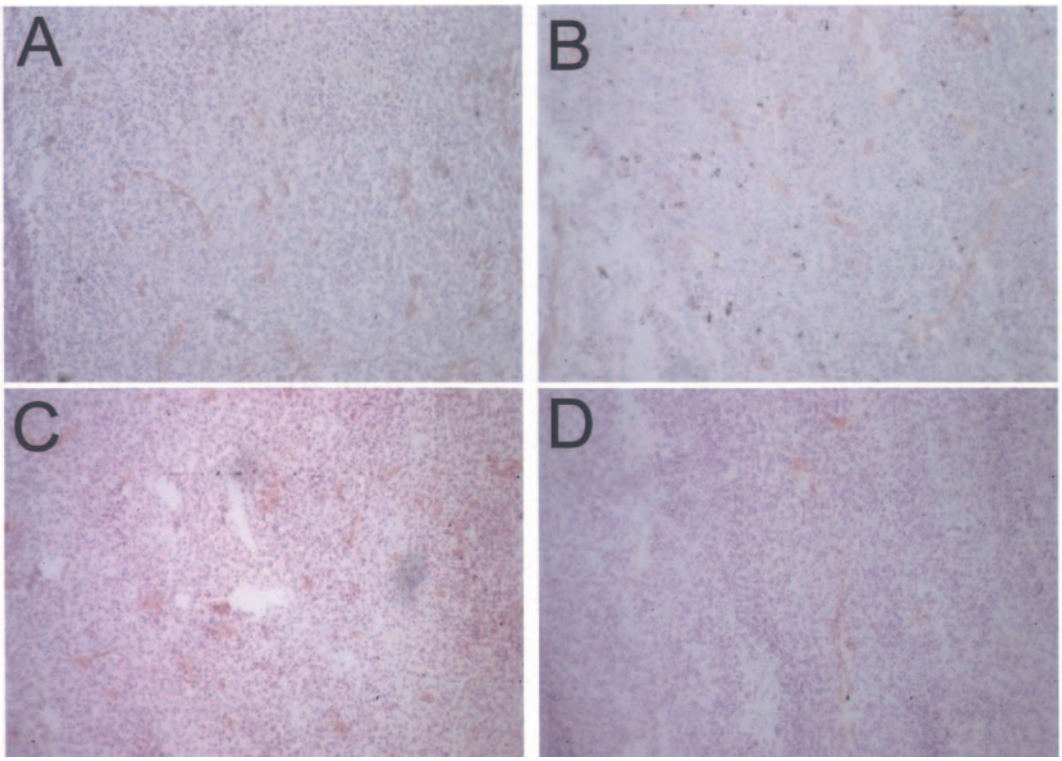


Figure 5. *Immunohistochemical staining for microvessels in glioma tissues. Representative photomicrographs of immunohistochemical staining for CD31 in the gliomas of the rats at week 4 after tumor cell inoculation. (A) no treatment; (B) treatment with propylene glycol; (C) treatment with 10 mg/kg/day of resveratrol; (D) treatment with 40 mg/kg/day of resveratrol. Positive staining represents the microvessels (x100).*

gliomas had a parallel level of macroscopic and microscopic angiogenesis. In addition, both the CDVI and MVD were significantly correlated with the tumor size ($r=0.794$ and 0.567, respectively, $p<0.0001$), indicating that both macroscopic and microscopic angiogenesis were correlated with tumor growth.

## Discussion

Resveratrol has been demonstrated to inhibit the proliferation of several kinds of malignancies (4-7, 9-13, 16). In recent years, resveratrol has further been found to inhibit angiogenesis in tumors, including gliomas, as revealed by the MVD measured from immunohistochemical staining (15-19). The MVD is typically used to study tumor-induced angiogenesis, however, it only reveals microscopic angiogenesis and not the global features of macroscopic angiogenesis or the degree of functionality of blood vessels (21-23). CDUS, on the other hand, can reveal large vessels in a tumor as well as the global features of macroscopic angiogenesis (24-32). The data from ultrasound examination can provide better information regarding microvessel density in the living state, usually not detectable in surgically-fixed specimens (25). These 2 methods are different but related (25, 31, 32), CDUS examination being complementary to immunohistochemical staining for MVD. In this study, both CDUS and immunohistochemistry were employed to investigate the effects of resveratrol on angiogenesis in gliomas.

CDUS was used to longitudinally monitor angiogenesis in gliomas along with the tumor growth of the individual glioma. It was found that the glioma CDVI increased gradually after tumor cell inoculation, but 40 mg/kg/day of resveratrol treatment significantly decreased the CDVI in weeks 3 and 4 after tumor cell inoculation. Furthermore, the CDVI was found to be significantly correlated with the tumor growth rate, indicating that the tumor growth in gliomas is related to macroscopic angiogenesis and that the inhibition of the tumor growth by resveratrol is related to the suppression of this macroscopic angiogenesis. The CDVI also correlated with the MVD for the same glioma and it was noted that high-dose resveratrol suppressed both the CDVI and MVD in weeks 3 and 4, with significant correlation between these data. These results suggest that both macroscopic and microscopic angiogenesis, represented by the CDVI and MVD, respectively (21, 31), increased after glioma cell inoculation, but that high-dose resveratrol (40 mg/kg/day) was able to inhibit both macroscopic and microscopic angiogenesis. The correlation between tumor size and the CDVI or MVD was further analyzed and it was found that tumor growth was correlated with both parameters, indicating the relationship between the tumor growth rate and both macroscopic and microscopic angiogenesis. In general, the MVD reveals multiple dilated vessels, as well as innumerable smaller vessels and microvessels, about 15 to 50 μm, from the periphery to the central portion of a tumor (21). In contrast, CDUS reveals larger vessels, possibly intratumoral arterioles and venules, as well as arteriole-venule shunting within the tumor; the examination often detects a single large vascular ring around the tumor, which represents a summation of the signal for these 100-mm adjoining vessels (21, 35). Gliomas usually have a rich peripherally-located blood supply, with small vascular channels penetrating the tumor (36). The significantly correlated CDVI, MVD and tumor size suggest that both microscopic and macroscopic angiogenesis in gliomas change proportionally along with tumor growth. Thus, the combination of both the MVD and CDVI methods, demonstrating both small and large vessels, might more clearly reveal the general vessel distribution in the individual glioma. This report is the first to demonstrate that the anti-angiogenic effect of resveratrol on gliomas is correlated with the inhibition of tumor growth.

Both the immunohistochemical and CDUS techniques, including CDUS images and histological slices, might not be representative of the tumor as a whole (28, 37): it is not easy to obtain a perfect match between sonographic and histological section plates under *in vivo* conditions (37); due to tumor vessel heterogeneity, there can be a selection bias for the area of most intense neovascularization in MVD calculations (28); the targeting of blood vessels in ultrasound measurement can cause inter- and intra-observer variability (29); and CDUS is technique-dependent with the data affected by motion artifacts (25, 29, 31). All these factors might influence the MVD or CDVI data or both. Overall, we can conclude that the combination of these 2 methods increases the accuracy of the study of tumor-induced angiogenesis since they represent different aspects of tumor-related angiogenesis.

CDUS was previously used mainly for diagnostic purposes and for correlations with patient survival (37). Only a few studies have used CDUS to assess the effects of chemotherapy on tumor blood flow (37). Because CDUS can be performed easily, non-invasively and serially for the measurement of tumor vascularity (21, 31), it might be useful as an *in vivo* method to assess tumor angiogenesis. On the other hand, the MVD is determined retrospectively and *in vitro* and, therefore, does not play a role in the preoperative evaluation and treatment planning for patients with neoplasms (28). By combining these 2 methods in clinical practice, CDUS could be used to improve the pre-operative understanding of tumor vascularity and blood flow, while immunohistochemical staining of the endothelial cells (MVD) could be employed to assess the tumor microangiogenesis in the surgical specimen. CDUS could

also be used in post-operative follow-up to monitor for tumor recurrence and evidence of angiogenesis. From the practical point of view, it is difficult to apply CDUS to the study of intracranial gliomas because the skull is a barrier to ultrasound. However, the development of transcranial Doppler (TCD) to study intracranial vessels (38) may lead to a solution to this problem. In the future, if CDUS could be used to monitor angiogenesis of intracranial tumors, neovascularization, as demonstrated by angiographic examination and immunohistochemical staining of the surgical specimen, could lead to a more complete understanding of both the microscopic and macroscopic features of induced angiogenesis in cerebral gliomas.

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