

## Resveratrol inhibits cell growth and induces apoptosis of rat C6 glioma cells

Wei Zhang · Zhou Fei · Hai-ning Zhen ·  
Jian-ning Zhang · Xiang Zhang

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**Abstract** Resveratrol (Res) has been reported to inhibit tumor initiation, promotion, and progression in a variety of cell culture systems depending on the specific cell type and cellular environment. In the present study, we determined the effect of Res on the cell growth and apoptosis of rat glioma C6 cell line as well as mouse fibroblast 3T3 cell line, *in vitro*. Concurrently, we investigated whether caspase-3 is involved in the Res-induced apoptosis of rat glioma cells. Exposure to Res exhibits a significant anti-proliferative effect and induces an increase in the population of apoptotic cells on C6 cells in a concentration- and time-dependent manner, but not for normal 3T3 fibroblast cells, as measured by methyl thiazolyl tetrazolium assay and flow cytometer. Distinguished increase of C6 cells in S phase is observed after the treatment of Res as compared to insignificant change in cell cycle distribution of 3T3 cells. TdT-mediated dUTP nick end labeling fluorescence staining, HE staining, and scanning electron microscope revealed abnormal morphology and ultrastructure in C6 cells treated with Res. Our data showed that Res can increase the expression and induced the activation of caspase-3 in rat glioma C6 cells. These results suggest that Res has significant apoptosis-inducing effect on C6 glioma cells other than normal fibroblast 3T3 cells *in vitro* and caspase-3 may act as a potential mediator in the process.

**Keywords** Resveratrol · Glioma · Cell cycle · Apoptosis

### Introduction

Glioma is the most malignant of primary brain tumors and carries the worst clinical prognosis in both adults and children, but effective therapy for such tumors is limited by their resistance to conventional ones. Till now, the mainstay of treatment is surgical debulking and radiotherapy, however, the overall survival remains poor. Several chemotherapeutic agents have been used in clinic, since most of them kill tumor cells via apoptosis, therefore, it is essential for us to understand the molecular signaling mechanisms of apoptosis in glioma cells in aim to exploring more effective therapeutic strategies with less toxicity [1].

Resveratrol (Res) (3,5,4'-trihydroxy-*trans*-stilbene), which is originally identified as a phytoalexin and is abundant in grapes, peanuts, pines, and other Leguminosae family plants in response to injury, ultraviolet (UV) irradiation, and fungal attack, has been reported to exhibit a variety of important biological effects such as the potential role to mediate strong anti-oxidant, anti-mutagenic, and anti-inflammatory effect [2]. Recently, several studies showed that Res inhibited the growth of different human cancer cell lines, including human oral squamous carcinoma, promyelocytic leukemia, breast, prostate, and colon cancer cells [3–7]. From then on, the potent cancer chemopreventive effects of Res in carcinogenesis become appealing.

The anti-carcinogenesis activity of Res was first demonstrated in a pioneering study by John Pezzuto and his colleagues, who reported that Res was effective

W. Zhang · Z. Fei · HN. Zhen · JN. Zhang ·  
X. Zhang (✉)

Department of Neurosurgery, Neurosurgical Institute of Chinese PLA, Xijing Hospital, Fourth Military Medical University, No. 127 West Chang'le Road, Xi'an 710032, Shaanxi Province, People's Republic of China  
e-mail: xzhang@fmmu.edu.cn

in all three major stages (initiation, promotion, and progression) of carcinogenesis [2]. Accumulating data from many cell line studies indicated that Res possessed strong anti-proliferative and apoptosis-inducing properties. S-phase or G<sub>1</sub>-phase arrest was mostly observed in Res-induced apoptosis, but the apoptosis-inducing effects of Res appeared diverse on different tumor cells [8–15]. These conflicting results of Res-induced anti-tumor biological impacts might be due to the specific cell type and cellular environment. As to glioma cells, whether Res exerted apoptosis effects on this type of cells as well as the underlying mechanisms remained largely unknown [16].

Caspase-3 has long been believed to correlate with apoptosis and survival. Since it represents the last step in the cascade to apoptosis before nuclear fragmentation, its activation has been supposed to be closely related to apoptosis of tumor cells [17]. Therefore, in the present study, reverse transcriptase-polymerase chain reaction (RT-PCR) and Western blot were used to determine the mRNA expression and cleaved protein of caspase-3.

The current study was undertaken to determine the contribution of Res to the proliferation and apoptosis of glioma C6 cell line as well as normal fibroblast 3T3 cell line *in vitro* and the possible involvement of caspase-3 was also investigated with Western blot on caspase-3 expression and activation after Res treatment.

## Materials and methods

### Cell lines and culture

The cell lines used in this study were the rat C6 glioma cells and the mouse NIH 3T3 fibroblast cells which were served as control. The two lines were purchased from ATCC. All the two cell lines were maintained in DMEM (Dulbecco's modified Eagle's medium, Gibco Life Science, Grand Island, NY, USA) supplemented with 10% of FBS (fetal bovine serum, Gibco Life Science, Grand Island, NY, USA), 2 mM of L-glutamate, 100 units/ml of penicillin, and 100 µg/ml of streptomycin. All cells were cultured at 37°C in a 5% CO<sub>2</sub> incubator.

### Inhibition effect of Res on cell growth

The sensitivity of the C6 cells as well as 3T3 cells to Res (Sigma Chemical Co., St Louis, MO, USA) was determined *in vitro* by an 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT)-based colorimetric assay.

For this purpose,  $2 \times 10^4$  cells were seeded in flat-bottomed 96-well plate (Nunc, Roskilde, Denmark), and cultured for 24 h to reach 60–70% confluence before Res treatment. Res was dissolved in dimethylsulfoxide (DMSO; Sigma, St Louis, MO, USA) to a stock concentration of 300 µM and diluted with culture medium to final concentrations of 30, 60, 90, 120, 150, 180, and 210 µM just before use. The C6 cells were exposed to the above-mentioned concentrations of Res for 0, 24, 48, 72, and 96 h, respectively. Each concentration group included eight wells. 3T3 cells received the same treatment to the C6 cells. The routinely cultured cells were used as normal control, in the medium containing the same concentration of DMSO as background control. The extent of the cell proliferation and cell viability was then determined by MTT assay. Briefly, cells were treated with 10 µl/well of MTT reagent for 4 h at 37°C and then treated with 100 µl/well of solubilization solution at 37°C overnight. The optical density (OD) value was measured by using a spectrophotometric microtiter plate reader (DG-3022, Shanghai Huadong Tube Factory, China) at 490 nm wavelength. The cell growth curves were drawn according to the measured OD value of each group. All experiments were performed in triplicate.

### Cell cycle analyses by flow cytometry

Analysis of cell cycle was performed by flow cytometric staining of permeabilized cells with propidium iodide (PI) for DNA content according to the manufacturer's instructions. Briefly, cells were plated in 10-cm culture dishes at concentrations determined to yield 60–70% confluence within 24 h. Cells were then treated with 0.1% DMSO or Res (210, 120, 0 µM). After 24 h of treatment, both adherent and floating cells were harvested and labeled with PI. Briefly, cells were re-suspended in phosphate-buffered solution (PBS), fixed with 70% ethanol, labeled with PI (0.05 mg/ml), incubated at room temperature in the darkness for 30 min, and filtered through 41-µm spectra/mesh nylon filters (Sigma, St Louis, MO, USA). DNA content was then analyzed using a FACScan instrument equipped with FACStation running Cell Quest software (Becton Dickinson, San Jose, CA, USA). All experiments were performed in triplicate and yielded similar results.

### Apoptosis assays by flow cytometry

The percentage of cells actively undergoing apoptosis was determined using annexin V-PE-based immunofluorescence. Briefly, cells were plated in 10-cm culture

dishes at concentrations determined to yield 60–70% confluence within 24 h. Cells were then treated with 0.1% DMSO or Res (210, 120, 0  $\mu$ M). After 24 h of treatment, both adherent and floating cells were harvested and then the Annexin V-FITC/PI Staining Kit (Immunotech Co., Marseille Cedex, France) was used according to the manufacturer's instructions. Cells were analyzed using a FACScan instrument equipped with FACStation running Cell Quest software (Becton Dickinson). PI negative and annexin V positive cells were considered as early apoptotic; cells that were both PI and annexin V positive were considered as in the late necrotic stage; cells that were PI positive and annexin V negative were considered as mechanically injured during the experiment; cells that were both PI and annexin V negative were considered normal. Experiments and control experiments were performed third times on different days using the same protocol and time exposures.

#### TdT-mediated dUTP nick end labeling staining

DNA fragmentation was visualized by using the Cell Death Detection Kit (Fluorescein, POD, Roche Applied Science, Mannheim, Germany). The cell-bearing cover-slips, which were treated with 210- $\mu$ M Res for 24 h, were collected and fixed in cold acetone or 4% paraformaldehyde in PBS (pH 7.4) before TdT-mediated dUTP nick end labeling (TUNEL) fluorescence staining. TUNEL fluorescence staining was then conducted with all procedures performed strictly according to the manufacturer's instructions. Afterwards, signal conversion was followed. Briefly, 50- $\mu$ l converter-POD was added on each slide and then slides were incubated in a humidified chamber for 30 min at 37°C, after the slides were rinsed with PBS for three times, 50- $\mu$ l DAB substrate was added on each slide and incubated for 10 min at 15–25°C, followed by the slides were mirror results rinsing with PBS for three times, the cell-bearing cover-slips was mounted and analyzed under light microscope.

#### Morphology and ultrastructure

To observe the morphological changes of apoptotic cells by Res, the cell-bearing cover-slips which were treated with 210- $\mu$ M Res for 24 h were then subjected to HE staining. In parallel, the same treated cell-bearing cover-slips were fixed for 1 h at room temperature with 4% glutaraldehyde in PBS and proceeded for scanning electron microscopy (S-570, Hitachi, Tokyo, Japan) as routine methods.

#### Reverse transcriptase-polymerase chain reaction

At the designated time, both adherent and floating cells were harvested. Cells were lysed in 1 ml of TRIzol Reagent (Gibco BRL, Grand Island, NY, USA). RNA was then extracted by adding 0.2 ml of chloroform and then precipitated with 0.5 ml of isopropanol. After centrifugation, the pellet was washed with ice-cold 75% ethanol, dried, and dissolved in RNasefree water (Promega, Mannheim, Germany). The RNA concentration was determined by measuring the absorbance at 260 nm. The purity of RNA was estimated by the ratio of A260 nm/A280 nm and was found to be 1.7–2.0. The first strand of cDNA was reversely transcribed with M-MLV RT (Gibco BRL, Grand Island, NY, USA) and oligo (dT)12-18 primer (Gibco BRL, Grand Island, NY, USA) by random-priming at 37°C for 60 min, and terminated by heating at 65°C for 5 min. The synthesized first strand cDNAs were amplified by using primers specifically designed for the caspase-3 receptor.  $\beta$ -Actin was served as an endogenous internal standard control for variations in RT-PCR efficiency. The caspase-3 primers were designed according to caspase-3 mRNA sequence (GeneBank accession number U26943, National Center for Biotechnology Information, USA) and were sense 5'-TTT GTT TGT GTG CTT CTG AGCC-3' and anti-sense reverse: 5'-ATT CTG TTG CCA CCT TTC GG-3', yielding a 400-bp product.  $\beta$ -Actin sequences were sense 5'-TGG TGG GTA TGG GTC AGA AGG ACTC-3' and anti-sense 5'-CAT GGC TGG GGT GTT GAA GGT CTC A-3', yielding a 265-bp product. PCR reactions were carried out in 50  $\mu$ l of reaction mixture containing 10 mM of Tris (pH 8.3), 50 mM of KCl, 1.5 mM of MgCl<sub>2</sub>, 1 ml of synthesized first strand, 40 pmol of each 50 and 30 primer pair, 0.1 mM of each dNTP and 2.5 units of Taq DNA polymerase (Takara, Shiga, Japan). The PCR reaction was performed for 30 cycles by using a PTC-100 Programmed Thermal Controller (MJ Research, Watertown, MA, USA) as follows: denaturation at 93°C for 1 min, annealing at 56°C for 30 s, followed by an extension at 72°C for 8 min. All experiments included reverse transcription negative controls where RT was omitted to exclude the possibility of genomic DNA amplification. After amplification, PCR products were separated on a 2% agarose gel containing ethidium bromide. For evaluation of changes in caspase-3 mRNA, gel bands were visualized in a UV-transilluminator and images were captured by using an 8-bit CCD camera (Ultra-Violet Products, Cambridge, UK). The relative gray levels of the bands, expressed as OD units, were quantitatively analyzed by

using Labworks Image Software (Ultra-Violet Products, Cambridge, UK). In all cases, reverse transcription negative controls were not amplified and could not be detected on the gel. The relative intensity of each band of the RT-PCR product (caspase-3) was normalized after dividing by the relative intensity of the corresponding band of  $\beta$ -actin.

#### Western blot analysis (protein determination)

To determine the receptor protein expression following Res treatment,  $2.5 \times 10^5$  C6 glioma cells were seeded onto each 100 mm diameter dish followed with the treatment with Res. Fifty micrograms membrane preparations were loaded on a 10% polyacrylamide gel.

After electrophoresis, proteins were transferred to a Hybond-C membrane (Amersham, Buckinghamshire, UK). The membrane was probed with a polyclonal antibody against caspase-3 (1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), or an anti-rabbit actin antibody (1:2,000, Santa Cruz Biotechnology). The primary antibody was then labeled with a horseradish peroxidase-conjugated anti-rabbit antibody (secondary antibody, 1:1,000, Santa Cruz Biotechnology). The blots were then developed by using the enhanced chemiluminescence detection method (Amersham, Buckinghamshire, UK). The densities of the protein blots were analyzed by using Labworks Software (Ultra-Violet Products). The relative protein densities under different experimental conditions were calculated as percentage of that for actin blot.

#### Statistical analyses

Data are expressed as mean  $\pm$  SD. Comparisons between DMSO-treated control cells and Res-treated cells were made using ANOVA.  $P < 0.05$  between groups was considered statistically significant.

## Results

#### Cell growth inhibition effect of Res in C6 glioma cells

To comprehend the possible inhibitory effect of Res on C6 cells as well as 3T3 cells, MTT assay was conducted as described above. Figure 1a revealed the survival curve of the C6 glioma cells treated with increasing concentrations of Res for different exposure times. Generally, the higher the Res dosage and the longer the drug exposure time, the greater the proportion of cells that were killed ( $P < 0.05$ ). However, in 96-h

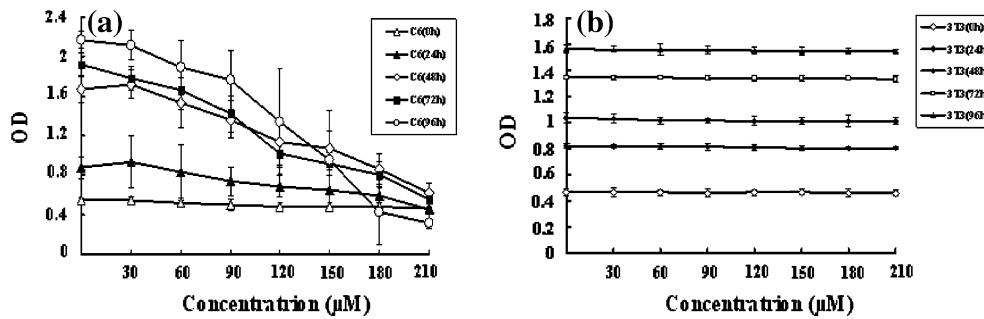
group, there was no statistically significant of cell growth inhibition between 180 and 210  $\mu$ M groups ( $P > 0.05$ ). Therefore, after sufficient exposure time, C6 cells might be insensitive when the concentration of Res reached a certain high level. According to our study, the critical point lied on 180  $\mu$ M. Figure 1b showed the survival curve of 3T3 fibroblast cells using the corresponding dosages and times as C6 glioma cells received. Of note, the proliferation of 3T3 cells was not suppressed by the diverse concentrations of Res for increasing exposure times ( $P > 0.05$ ). Thus, our data first demonstrate that Res exerts a significant cell growth inhibition effect upon glioma C6 cells in a concentration- and time-dependent manner, but not for normal 3T3 fibroblast cells.

#### Res induces S-phase cell cycle arrest in C6 glioma cells

In search of a potential basis for the observed cell growth inhibition by Res, we performed flow cytometry analysis on cell cycle progression. Cells were treated with DMSO (0.1%) alone or with 0, 120, 210  $\mu$ M of Res. Since we were interested in evaluating the distribution of actively dividing cells before the induction of extensive apoptosis, we harvested cells at 24 h, rather than at 48 h as many studies did. After 24 h of treatment, cells were labeled with PI and analyzed by DNA flow cytometry. The data obtained from two cell lines were summarized in Tables 1 and 2, respectively. We observed more than a twofold increase in the number of C6 cells in S phase after treatment with 210- $\mu$ M Res for 24 h as compared with the controls (Fig. 2). Interestingly, there was no significant change in cell cycle distribution of normal 3T3 fibroblast cells.

#### Apoptosis of the glioma cells induced by Res

In view of the inhibition of cell cycle progression, we were interested in determining whether Res also induced apoptosis in the two cell lines in the early stage of the treatment, we harvest cells at 24 h, rather than 48 h as many other studies did. The cells were treated with 0.1% DMSO alone or 0, 120, 210  $\mu$ M Res for 24 h. Representative results for the C6 cells and 3T3 cells were shown in Fig. 3. In C6 cells, the percentage of apoptotic cells were  $2.5 \pm 0.49\%$  ( $n = 3$ ),  $2.33 \pm 0.6\%$  ( $n = 3$ ),  $15.1 \pm 1.5\%$  ( $n = 3$ ), and  $29.9 \pm 2.61\%$  ( $n = 3$ ), respectively (Fig. 4); in contrast, the corresponding rates for 3T3 cells were  $3 \pm 0.46\%$  ( $n = 3$ ),  $3.1 \pm 0.31\%$  ( $n = 3$ ),  $3.2 \pm 0.3\%$  ( $n = 3$ ), and  $3.73 \pm 0.49\%$  ( $n = 3$ ). Thus, Res has significant



**Fig. 1** The effects of Res on cell growth—(a) growth curves of C6 cells treated for 0, 24, 48, 72, 96 h with increasing concentrations of Res. Among each group, 210 μM Res treated

for 96 h had most significant suppressing effect; (b) growth curves of 3T3 cells treated with Res for 0, 24, 48, 72, 96 h under various concentrations

**Table 1** Cell cycle analysis of C6 cells treated with Res

Cell cycle status	G <sub>0</sub> /G <sub>1</sub> phase	S phase	G <sub>2</sub> /M phase
DMSO (0.1%) control	79.6	15.8	4.6
0 μM	78.8	15.8	5.4
120 μM	77.8	19.6	2.6
210 μM	58.3	36.5	5.2

**Table 2** Cell cycle analysis of 3T3 cells treated with Res

Cell cycle status	G <sub>0</sub> /G <sub>1</sub> phase	S phase	G <sub>2</sub> /M phase
DMSO (0.1%) control	79.8	15.4	4.8
0 μM	79.3	15.6	5.1
120 μM	79.2	15.8	5.0
210 μM	78.8	15.5	5.7

apoptosis-inducing effect on C6 glioma cells other than normal fibroblast 3T3 cells in vitro.

Morphology and ultrastructure of apoptotic cells

Since the potential growth inhibition and apoptosis-inducing effects of Res against C6 cells were remarkable, the morphological changes were examined. As

illustrated in Fig. 5, cells exposed to Res exhibited the distinct apoptotic features, such as nuclear swelling, karyopycnosis, karyorrhexis, cell shrinkage, and cell membrane blebbing.

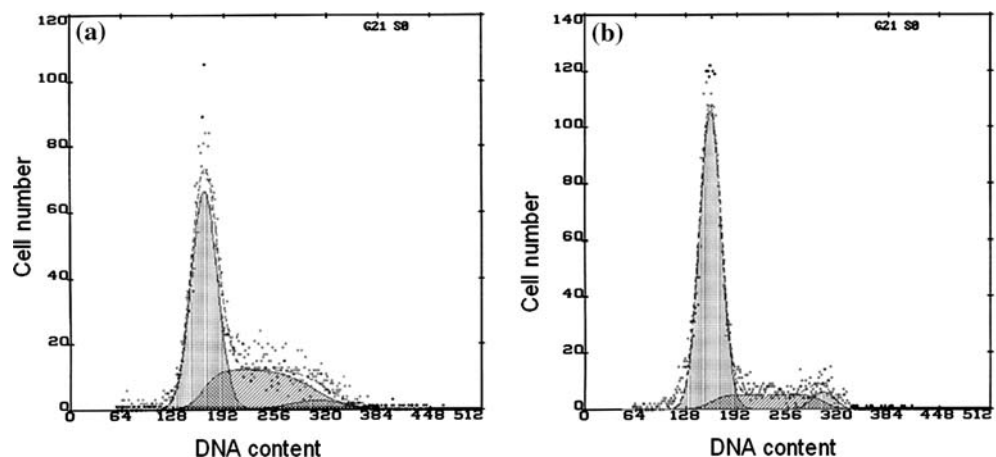
Up-regulation of caspase-3 mRNA expression in C6 glioma cells following the treatment of Res

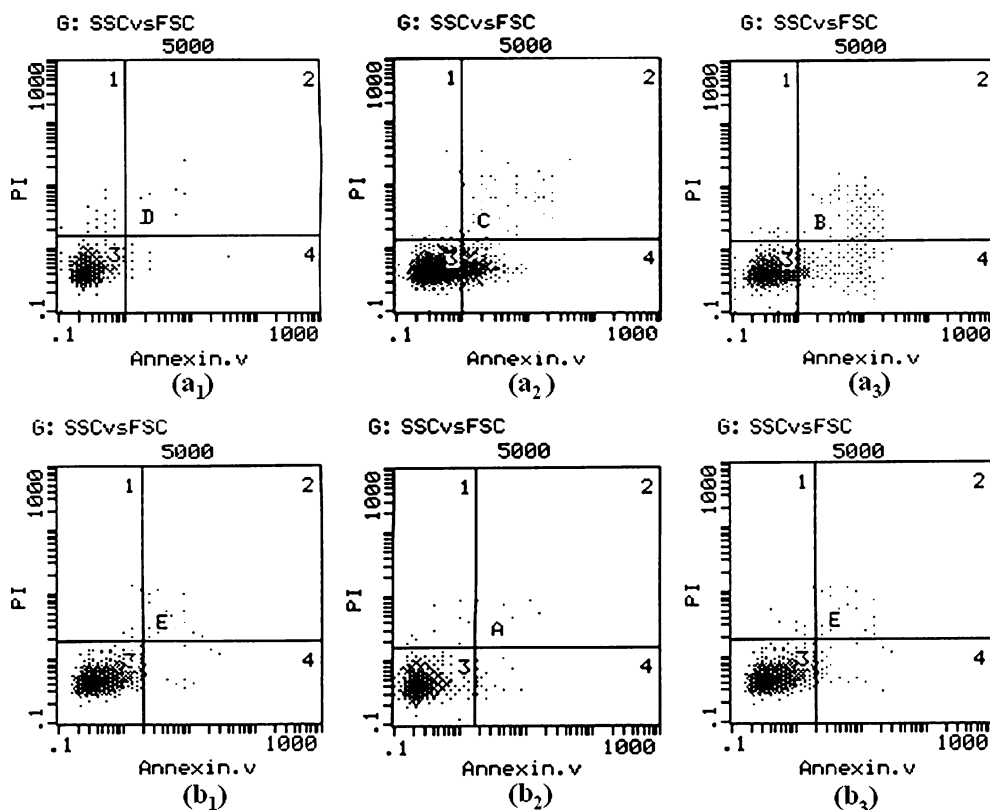
As Fig. 6a demonstrated, cells treated with 120 or 210 μM Res (24 and 48 h) showed marked increase in caspase-3 mRNA expression. When compared with the samples taken from 0.1% DMSO group, 24 and 48 h after the treatment of Res at the concentration of 120 or 210 μM, the expression of caspase-3 mRNA significantly increased ( $P < 0.05$ ). The cells harvested at 48 h after the treatment of 210 μM Res exhibited the highest expression of caspase-3 mRNA (Fig. 6b). There were little changes in mRNA level between the cell samples treated with 0.1% DMSO and 0 μM Res.

Effect of Res on caspase-3 activation

A band of procaspase-3 (approximately 32 kDa) was detected in control and Res-treated tumor cells;

**Fig. 2** Cell cycle analyses by flow cytometry—(a) cell cycle analysis of C6 cells after treatment with 210 μM Res for 24 h. S-phase arrest was significant compared with the 0.1% DMSO control group; (b) cell cycle analysis of C6 cells after treatment with 0.1% DMSO for 24 h

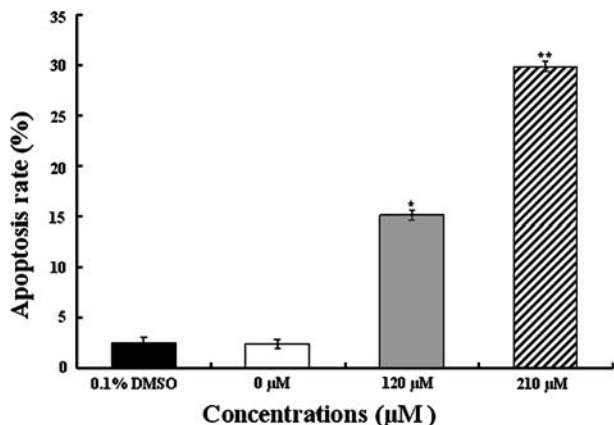




**Fig. 3** The apoptosis-inducing effect of Res on C6 cells and 3T3 cells. C6 (**a<sub>1</sub>**, **a<sub>2</sub>**, **a<sub>3</sub>**) and 3T3 (**b<sub>1</sub>**, **b<sub>2</sub>**, **b<sub>3</sub>**) cells were treated with 0.1% DMSO or Res (120, 210  $\mu$ M) for 24 h

however, the 17-kDa band, which corresponds to active caspase-3, is observed exclusively in C6 glioma cells treated with Res (Fig. 7a). The present data shows a markable increase in binding density of procaspase-3 in C6 glioma cells treated with Res in a concentration- and time-dependent manner (Fig. 7b;  $P < 0.05$ ). Concurrently, Res induced the generation of cleaved

caspase-3 after 24 h of 120- $\mu$ M Res treatment. There is considerable increase in active caspase-3 induction at 48 h under the treatment of 210- $\mu$ M Res, when compared to the concentration of 120- $\mu$ M Res (Fig. 7c,  $P < 0.01$ ).



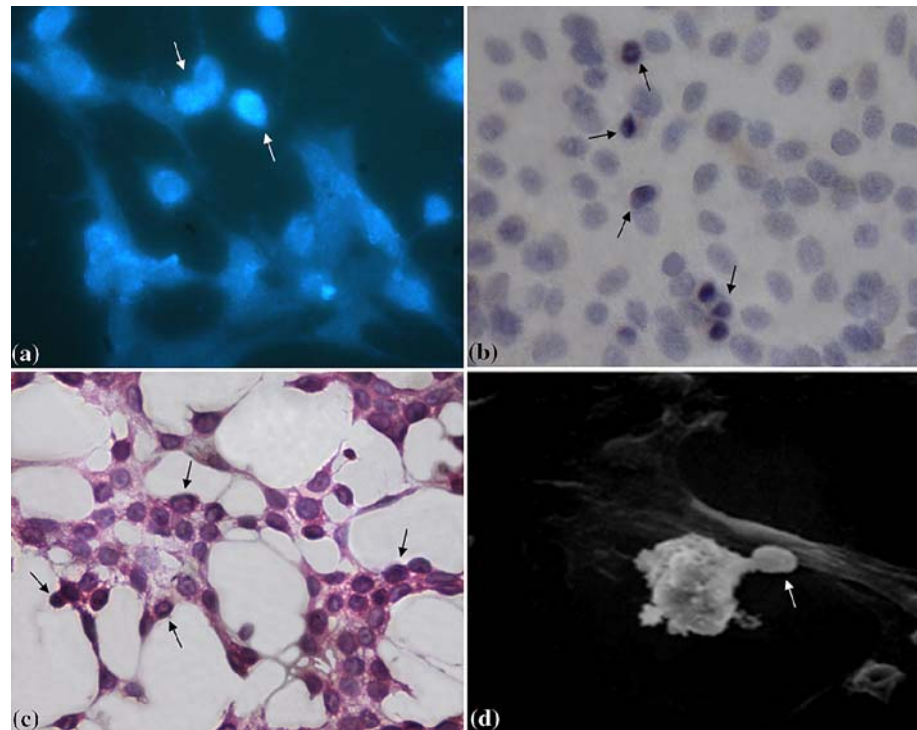
**Fig. 4** Apoptosis rate of C6 cells by flow cytometry after treated with DMSO (0.1%) alone or increasing concentrations of Res for 24 h. The 120 and 210  $\mu$ M of Res showed significantly increased apoptosis rate compared with the controls ( $P < 0.05$ )

## Discussion

Chemoprevention, a relatively new strategy to prevent cancer, depends on the use of nontoxic chemical substances to block, reverse, or retard the process of carcinogenesis and keen interests have been focused on plant-based diet as potential chemopreventive agents [5].

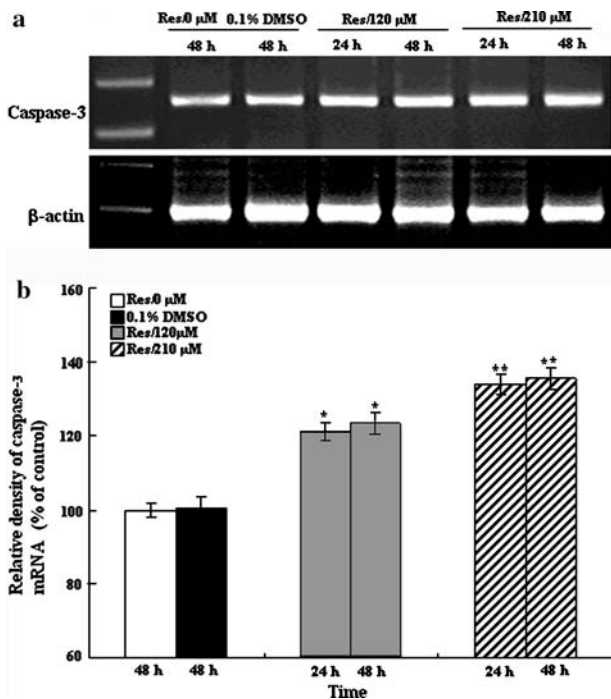
Resveratrol (3,5,4'-trihydroxy-*trans*-stilbene) is a naturally occurring polyphenol synthesized by a variety of plant species in response to injury, UV irradiation, and fungal attack [18]. Accumulating evidences indicated that an inverse relationship existed between Res and its striking inhibition of diverse cellular events associated with tumor initiation, promotion, and progression [8, 11, 19]. Res has now been recognized as a promising candidate of chemopreventive effect.

**Fig. 5** Morphological changes in cultured C6 cells treated with 210  $\mu\text{M}$  Res for 24 h—(a) investigation of apoptotic cells by TUNEL fluorescence staining, the positive cells presented *bright blue* color in nucleus ( $\times 400$ ); (b) after signal conversion, apoptotic cells by TUNEL fluorescence staining were observed under light microscope. The positive cells exhibited *deep brown* color in nucleus ( $\times 200$ ); (c) by HE staining, nuclear swelling, karyopycnosis as well as karyorrhexis (*arrow* indicated) were presented in apoptotic cells ( $\times 200$ ); (d) by scanning electron microscope, the apoptotic cells (*arrow* indicated) exhibited shrinkage and membrane blebbing ( $\times 2,500$ )



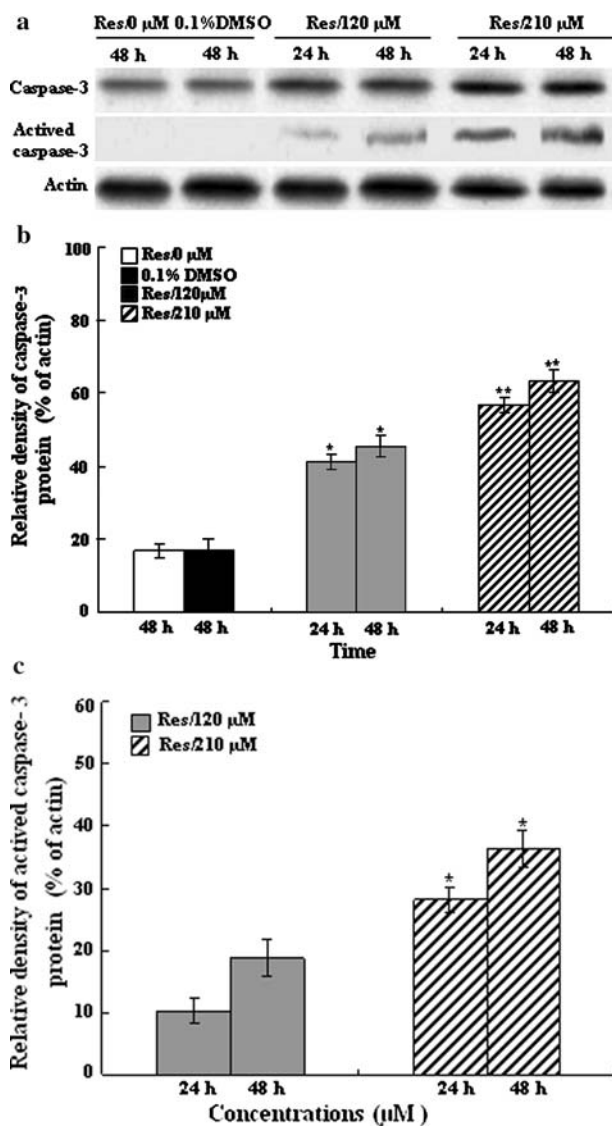
Diffuse malignant glioma is the most common type of brain tumor. Although the prognosis of intracranial tumors has been improved, the outcome of glioma

remains poor due to the difficulty in removing the invasive growth tumor radically and short- and long-term adverse effects of conventional post-surgical adjuvant therapies. Consequently, glioma has the highest death rate among the intracranial tumors.



**Fig. 6** Representative RT-PCR and histogram summary results from RT-PCR analysis of C6 cells treated with DMSO (0.1%) alone or increasing concentrations of Res for 24 or 48 h. (a) Sample RT-PCR showing expression of caspase-3 mRNA in the C6 cells; (b) relative density of caspase-3 mRNA normalized to cells treated with Res (0  $\mu\text{M}$ )

Accumulating data from various cell line studies showed that Res possessed strong anti-proliferative properties. G<sub>1</sub>-phase arrest was observed in human epidermoid carcinoma A431 cells [20], Hep G<sub>2</sub> cells [21], and esophageal adenocarcinoma Bic-1 [22]. However, most of the cell lines demonstrated an accumulation in S phase. The ability of Res to block the S-G<sub>2</sub> transition has been reported previously in HL60 leukemia [23], U937 lymphoma [24, 25], MCF-7 breast carcinoma [26], SK-Mel-28 melanoma [27], Seg-1 adenocarcinoma, HCE7 esophageal squamous carcinoma, SW480 colorectal carcinoma [22], and CaCo-2 colon cancer cells [6]. Our data reveal that a distinguished increase in S phase is caused in C6 glioma cells with 210  $\mu\text{M}$  Res treatment for 24 h. In agreement with our finding, the S-phase arrest by Res was also exhibited in human neuroblastoma SH-SY5Y cells [28] and neuro-2a neuroblastoma cells [19]. However, in other studies, Res-induced G<sub>1</sub> arrest was observed in glioblastoma U373MG, A172 cells [29], and human glioma U251 cells [16]. The mechanisms of Res-induced cell cycle inhibition were not consistent depending on cell types. Generally, it may be related with the up-regulation of cyclin E [19, 28], cyclin A, down-regulation of cyclin D1 [28], p21 [19], surviving [29], sensitization for



**Fig. 7** Representative Western blots and histogram summarized results from Western blot analysis of C6 cells treated with DMSO (0.1%) alone or increasing concentrations of Res for 24 or 48 h. **(a)** Sample Western blots showing expression of procaspase-3 and cleaved caspase-3 in C6 cells; **(b)** relative density of procaspase-3 protein normalized to actin; **(c)** relative density of cleaved caspase-3 protein normalized to actin

tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis [29], activation of caspase-3, and increasing the cleavage of the downstream caspase substrate [16]. Besides cell cycle arresting properties, apoptosis-inducing effect is another essential feature of Res. It has been previously shown to trigger apoptosis in leukemia, mammary, myeloma, epidermoid, embryonal rhabdomyosarcoma, and glioblastoma cell lines [8–12, 20]. The doses required for Res to induce apoptosis were often higher than those that induced growth inhibition and cell cycle arrest [23] and were

often in the 100–200  $\mu$ M range [13–15]. In this study, the primal highest concentration we chose was 300  $\mu$ M (data not shown) to convincingly demonstrate the ability of Res to induce apoptosis in C6 glioma cells, however, the cell growth result showed no statistically significant ( $P > 0.05$ ) between 300 and 210  $\mu$ M groups in each time point (24, 48, 72, 96 h). Therefore, 210  $\mu$ M was set as the highest level. In turn, we found that Res generally elicited a concentration- and time-dependent inhibition of glioma cell proliferation. Particularly, C6 cells might be insensitive when the concentration of Res reached a certain high level. According to our study, the critical point lies on 180  $\mu$ M. We further explored the effect of Res on the induction of glioma cell apoptosis and noted that Res also induced apoptosis in glioma cells, as was seen in a variety of other cancer cells [8–12]. The observed Res-induced apoptosis of the C6 glioma cells appeared to occur in a concentration- and time-dependent manner, with higher concentrations and prolonged exposure eliciting significant cellular apoptosis. The precise mechanisms of Res-induced apoptosis in brain tumors still remain unclear. According to Wang et al., Res-induced Fas-independent apoptosis of human medulloblastoma cells in a time- and dose-related fashions [30]. Moreover, Jiang et al., showed that Res-induced apoptosis of glioma U251 cells was attributable to caspase activation and increased accumulation of Bax [16].

Caspase-3, one of the key caspase executioners, has long been believed to play important roles in apoptosis of tumor cells [17]. In the present study, we observed that the exposure to Res resulted in a significant increase of the expression of caspase-3 mRNA and procaspase-3 in C6 glioma cells in contrast to no marked changes in glioma cells treated with 0.1% DMSO. Activated caspase-3 was observed exclusively in C6 glioma cells with the treatment of Res at the concentration of 120 or 210  $\mu$ M. These data might indicate that apoptosis induced by Res is mediated through the activation of caspase-3.

The present study demonstrates that diverse concentrations and exposure time of Res did not suppress the cell growth and induce significant change in cell cycle distribution of normal fibroblast 3T3 cells. Meanwhile, Res has no apoptosis-inducing effect on 3T3 cells. This result appears particularly appealing, since the tumor-specific effect plays a crucial role in anti-glioma therapies.

Combined with in vitro studies, few researches explored the effects of resveratrol in vivo. Excitingly, Res slowed the growth of gliomas in rats, fibrosarcomas [31], Lewis lung carcinoma in mice, prevented tumor metastasis [32], reduced total number of tumors in rats



[33], prolonged animal survival time, and increased animal survival rate [34]. Thus, Res might have prosperous clinical perspective in the treatment of glioma.

However, the potential clinical implications of our studies will also depend on whether or not Res can be given safely to humans at doses high enough to achieve pharmacologically active effects. Thus, the combination of Res and other anti-glioma therapies may be a novel strategy for the treatment of glioma that deserves further investigation.

In summary, our study demonstrates that Res exerts a significant anti-proliferation effect against glioma C6 cells in a concentration- and time-dependent manner, but not for normal 3T3 fibroblast cells. A distinguished increase of C6 cells in S phase with the treatment of 210- $\mu$ M Res for 24 h was found, as for 3T3 cells, no significant change in cell cycle distribution is observed. It is notable Res has significant apoptosis-inducing effect on C6 glioma cells over normal fibroblast 3T3 cells in vitro and this effect might be mediated through the activation of caspase-3.

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