Full paper

Resveratrol, a potential radiation sensitizer for glioma stem cells both in vitro and in vivo

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1. Introduction

Glioblastoma (GBM) is a malignant human cancer that confers a dismal prognosis. Ionizing radiation (IR) is applied as the standard treatment for malignant gliomas. However, radiotherapy remains merely palliative because of the existence of glioma stem cells (GSCs), which are regarded as highly radioresistant “seed” cells. In this study, the effect and possible mechanisms of radiotherapy in combination with resveratrol (Res) were investigated in a radioresistant GSC line, SU-2. Our results showed that Res inhibited SU-2 proliferation and enhanced radiosensitivity as indicated by clonogenic survival assay. We also observed a decrease in the expression of neural stem cell marker CD133, which indicated that treatment with Res and IR induced SU-2 cell differentiation. In addition, the combination of Res with IR significantly increased autophagy and apoptosis levels in both in vitro cells and nude mouse model. Finally, Res significantly attenuated the repair of radiation-induced DNA damage. Taken together, the present study demonstrated that the significant radiosensitization ability of Res both in vitro and in vivo was attributed to its synergistic antitumor effects, including inhibition of self-renewal and stemness, induction of autophagy, promotion of apoptosis, and prevention of DNA repair. Therefore, Res may function as a radiation sensitizer for malignant glioma treatment.

Resveratrol (3,4',5-trihydroxystilbene, Res), a potential radiation sensitizer for tumor treatment, which is a naturally occurring phytoalexin enriched in enormous dietary products, has attracted research attention because of its cardioprotective, antioxidant, and anti-inflammatory activities and cancer chemopreventive properties (9,10). Recent studies have revealed that Res has growth inhibitory activity, and it induces apoptotic or autophagic cell death in a number of human cancer cell lines and in animal models of carcinogenesis (11–14). Res inhibits cell proliferation and stimulates apoptosis through activation of the mitochondrial apoptotic pathway in human retinoblastoma cells (15). Res can also induce ovarian cancer death through two distinct pathways: apoptosis and autophagy (16). Yu et al. suggested that Res can inhibit the activation of STAT3 signaling of medulloblastoma cells, and it may further induce medulloblastoma cells to growth arrest and apoptosis (17). Several groups have demonstrated that Res enhances radiation sensitivity in prostate cancer by inhibiting cell proliferation and promoting cell senescence and apoptosis (18,19). Hence, Res plays crucial roles in anti-proliferative, pro-apoptotic, and radiosensitizing effects on various cancer cells.

Previous studies in our laboratory have shown that Res exerts growth inhibitory effects on glioma U251 cells in a time- and dose-
2. Materials and methods

2.1. Cell culture

Human GSC line SU-2 was obtained from a surgical specimen of a patient with mixed tumors containing anaplastic astrocytoma and ependymal cells (21, 22). The cells were cultured at 37 °C in an atmosphere with 5% CO2 in DMEM/F12 (Gibco Life Technologies, Paisley, UK) in the presence of recombinant human fibroblast growth factor (bFGF; 20 ng/ml; Invitrogen), recombinant human epidermal growth factor (EGF; 20 ng/ml; Invitrogen), and N2 supplement (Gibco Life Technologies) according to protocols in the literature (22). The cells were then differentiated in the presence of serum or in the absence of bFGF and EGF.

2.2. Reagents

Res (Sigma, St Louis, MO, USA, Lot: R5010) was dissolved in dimethyl sulfoxide (DMSO; Sigma Aldrich, St Louis, MO, USA) to produce a 100 mM stock solution, which was aliquoted, stored at −20 °C, and diluted to the desired final concentration in DMEM/F12 at the time of use.

2.3. Radiation treatment and clonogenic survival assay

Cells or mice were irradiated with a 6 MV X-ray linear accelerator (model: PRIMUS, D. E., Siemens AG) (23). The number of colonies with at least 50 cells was counted. Surviving fraction (SF) was calculated as: mean colony count of plated cells × plating efficiency.

2.4. Immunofluorescence

Immunofluorescence, monodansylcadaverine (MDC) staining, and Hoechst 33258 staining were conducted as described previously (23). The anti-CD133 antibody (1:100; Millipore), secondary antibody conjugated to Alexa 488 (Invitrogen), and Hoechst 33258 (0.5 mg/ml; KeyGEN) were used in the study.

2.5. Western blot analysis

Western blot was performed as described previously (23). The following antibodies were used: rabbit anti-LC3 (1:1000; Abcam), rabbit anti-Beclin-1 (1:1000; Abcam), mouse anti-Bcl-2 (1:500; Abcam), and mouse anti-β-actin (1:1000; Cell Signal). Membranes were washed again with TBST and scanned with the Odyssey Infrared Imaging System (LI-COR).

2.6. Transmission electron microscopy (TEM)

The ultrastructure of SU-2 cells after treatment with or without ionizing radiation (IR) in the presence or absence of Res was determined by TEM as described previously (24). Tumor tissues obtained from mice bearing GSCs were cut serially into ultrathin sections and fixed with 2.5% glutaraldehyde at 4 °C for 2 h, followed by a second fixation with OsO4, ethanol dehydration, infiltration, embedding, and ultrathin sectioning by diamond knives. The slides were viewed by TEM with a Philips CM120 electron microscope at the voltage of 100 kV.

2.7. Annexin V-FITC/PI staining

The percentage of apoptosis was analyzed by flow cytometry with FITC/PI kit (KeyGEN, Nanjing, China) according to the manufacturer’s recommendations. Cells in the earlier stages of apoptosis were stained positive for annexin V-FITC, whereas those in the later stages of apoptosis were stained positive for both annexin V-FITC and PI.

2.8. Neutral comet assay

As a measure of DNA repair in SU-2 cells, the neutral comet assay was employed as previously described (23). DNA damage under each condition was quantified as follows: number of cells with the comet’s tail/number of total cells × 100%.

2.9. Animal experiments

All animal experiments were conducted in accordance with the humane treatment of animals under institutional guidelines approved by the Ethical Committee of Soochow University. The mice were housed in individually ventilated cages in the Animal Laboratory of the Medical College of Soochow University. Food and water were freely available.

Five-week-old male nude (BALB/c) mice were used in the present study. To generate tumor xenografts, 5 × 10^6 SU-2 cells in the logarithmic growth phase were implanted subcutaneously into the right forelimb of BALB/c nude mice. Xenografts were allowed to grow to approximately 100 mm³ over two weeks and randomly divided into four groups (n = 5 in each group): Con (100 μL, saline solution), IR (X-ray, 6 Gy), Res (150 mg/kg), and IR + Res (6 Gy, 150 mg/kg). Res were administered by intraperitoneal injection every other day for two weeks, and the IR treatment was given twice on day 3 and on day 9 with a dose rate of 2 Gy/min until 6 Gy IR had been delivered. Mice received local irradiation as described elsewhere (25).

From the day of intervention, the longest diameter (a) and shortest diameter (b) of the tumor were measured using digital calipers every 3 days, and the tumor volume (V = 0.5 × a × b²) was calculated. The individual relative tumor volume (RTV) was calculated as follows: RTV = Vt/V0, where Vt is the volume on each day, and V0 is the volume at the beginning of the treatment. The mental state, feeding, activity, urine, and feces of nude mice were observed during the experiment. At the end of the experiments, subcutaneous tumors were harvested following animal sacrifice by cervical dislocation. Tumor samples were excised for Western blot, fixed in 4% paraformaldehyde, and cut into 4 μm serial sections for TEM analysis.

2.10. Statistical analysis

All the experiments were repeated independently at least three times, and data were presented as the mean ± SD (standard deviation). Data were statistically analyzed using a two-tailed Student t-test. A p value of 0.05 was considered the boundary of statistical significance. All analyses were performed with GraphPad Prism 5.0.
3. Results

3.1. Res inhibits GSC proliferation and enhances radiosensitivity

Clonogenic assay was performed to determine whether the combination of Res and IR leads to radiosensitization of GSCs. We examined the effects of IR, Res, and IR + Res on cell survival. As a result, cells treated with Res or IR alone produced a reduced number of colonies compared with the group that was untreated, but the combination of Res and IR could generate a significantly reduced number of colonies compared with the group without treatment (Fig. 1A). Student’s t-test was performed on the SF values to demonstrate clonogenic assay studies. The analysis was performed by comparing SF of untreated (SF = 1) with SF of Res (SF = 0.85 ± 0.1), IR (SF = 0.81 ± 0.1), or IR + Res (SF = 0.2 ± 0.1) at 6 Gy. Cells previously treated with Res and IR generated a decreased number of colonies compared with untreated cells (p < 0.05, SF = 0.2 ± 0.1 for IR + Res) (Fig. 1B). These findings suggested that the combination of Res and IR significantly reduced the colony formation capacity of GSCs and significantly increased radiation sensitivity in GSCs.

3.2. Res attenuates self-renewal and reduces the stemness of GSCs

Self-renewal is a key feature of stem cells. The self-renewal ability of cancer stem cells (CSCs) is essential for tumorigenesis and tumor development (26). Therefore, we evaluated the effect of combined Res and IR on the self-renewal of GSCs based on the ability of GSCs to generate neurospheres. After cells were cultured in serum-free media with bFGF and EGF for a week, the number of neurospheres was counted to calculate the neurosphere formation rate: neurosphere formation rate = (number of neurospheres/number of inoculated cells) × 100%. Relative to cells in the control and IR-treated group, cells in the co-treatment group became more attached to the culture plate, losing their spherical shape and forming cell processes as indicated by arrows (Fig. 2A), and the neurosphere formation rate and size of the newly formed neurospheres in IR + Res group were remarkably decreased (Fig. 2B). The results suggested that the combination of Res and IR reduced the self-renewal ability of GSCs. To determine the effects of Res on the stemness of GSCs, we used immunocytochemical assay to detect changes in expression levels of neural stem cell marker CD133 after treatment for 48 h in various treatment groups. Compared with the control and IR groups, the expression of CD133 was reduced in the Res group. However, the level of CD133 in the co-treatment group almost disappeared, together with the loss of spherical shape (Fig. 2C). This result demonstrated that Res and IR reduced the expression of the neural stem cell marker CD133 of GSCs in vitro. Overall, our results indicated that combined Res and IR not only deregulated the self-renewal of GSCs but also reduced the stemness of GSCs. These results indicated that the combination of Res and IR

![Fig. 1. Resveratrol inhibits GSC proliferation and enhances radiosensitivity.](image-url)
enhanced radiosensitivity of GSCs might be through repressing self-renewal and reducing stemness of GSCs.

3.3. Res-mediated radiosensitization involves increased autophagy induction

Increasing evidence indicated that the radiosensitivity of cancer cells is associated with the induction of autophagy (21,27, 28). Therefore, we investigated whether Res-mediated radiosensitization of GSCs results from autophagy induction by performing MDC staining, Western blot, and TEM analysis. The autofluorescent agent MDC has been introduced as a specific autophagolysosome marker to analyze the autophagic process (29). We stained SU-2 cells with MDC and observed the staining patterns using a fluorescence microscope to monitor the autophagy/lysosomal events. After exposure of GSCs to IR, Res, or IR + Res, the SU-2 cells that were treated with Res alone or with Res and IR showed an increased number of MDC-positive vesicles in the cytoplasm, particularly in the perinuclear regions (Fig. 3A). This increase suggested the formation of autophagic lysosomes and an increase in

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**Fig. 2. Effect of resveratrol on self-renewal and stemness of GSCs.** (A) Representative images of SU-2 neurospheres after treatment with IR, Res, or both modalities (Scale bar = 50 μm). (B) The quantification of neurospheres formation rate and size of neurosphere formed under each condition. (C) Immunocytochemistry for the neural stem cell marker CD133 was performed in SU-2 neurospheres after the indicated treatments on coverslips (Scale bar = 20 μm). Nuclei were counterstained with DAPI. Mean ± SD, n = 3. **p < 0.001 compared with the control group. ***p < 0.001 compared with the IR-alone group.
the induction of autophagy. Induction of autophagy is usually accompanied with an increase in microtubule-associated protein light chain 3 (LC3) and Beclin-1. Another approach is to detect LC3 conversion (LC3-I to LC3-II) or Beclin-1 by immunoblot analysis, because the amount of LC3-II is clearly correlated with the number of autophagosomes (30). We then performed Western blot to analyze the changes in the protein expression levels of LC3-I, LC3-II, and Beclin-1 in GSCs with the same treatments as described above (Fig. 3B). The quantification of the percentage of LC3-I and LC3-II expression in each of the treatment groups showed a remarkable increase in the conversion of LC3-I to LC3-II in SU-2 cells that received combined treatment ($p < 0.001$) (Fig. 3C). Similarly, results in Beclin-1 were observed ($p < 0.01$) (Fig. 3C). Interestingly, the level of LC3-II/LC3-I and Beclin-1 in the co-treated group was much higher than that in the IR alone group ($p < 0.01$). TEM analysis showed a significant increase in double-membrane autophagosomes in the Res-treated group and in the combination group as indicated by arrows (Fig. 3D). Collectively, these data suggested that the mechanism of Res-mediated radiosensitization involved the induction of autophagy.

### 3.4. Induction of apoptosis of GSCs by co-treatment with Res and IR

Autophagy and apoptosis usually function as partners to induce cell death in various cancer cells (31–33). Hence, we investigated whether combination therapy can induce apoptosis in GSCs. We performed annexin V-FITC/PI staining to measure the percentage of apoptosis in each treatment group as mentioned above. Compared with the untreated group, the IR alone group and Res alone group showed a slight increase in apoptosis level; however, apoptosis in the co-treated cells was approximately thirtyfold greater than that in the matched untreated cells and eightfold greater than that in the IR alone group ($p < 0.001$ and $p < 0.01$, respectively) (Fig. 4A and B). Another corroborative assay, Hoechst 33258, which is used to stain DNA for evaluating the cell cycle and apoptosis, was performed to determine the incidence of apoptosis morphologically. Representative microphotographs showed that morphological characteristics of apoptosis, such as chromatin condensation and nuclear fragmentation, were obvious in SU-2 cells treated with both Res and IR after evaluating stained nuclei by Hoechst 33258 for 48 h (Fig. 4C). All of these data collectively indicated that radiation sensitization in co-treated GSCs was due to higher rates of apoptosis.

### 3.5. Res impairs the repair of radiation-induced DNA damage in GSCs

CSCs may contribute to glioma radioresistance through preferential activation of the DNA damage checkpoint response and an increase in DNA repair capacity (34). In the present study, DNA

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**Fig. 3. Resveratrol-mediated radiosensitization involves autophagy induction.** (A) SU-2 cells were treated with Res (75 μmol/L) for 12 h and then harvested 24 h after treatment with IR (6 Gy), and the formation of autophagolysosomes was analyzed using MDC by immunofluorescence analysis (Scale bar = 10 μm). (B) The cells were treated with Res and IR and analyzed for LC3, Beclin-1, and β-actin expression by Western blot analysis. β-actin was used as a loading control. (C) Quantification of LC3 and Beclin-1 protein expression in SU-2 cells treated by indicated treatments after normalization with β-actin levels. (D) Representative electron micrograph image showing increased formation of autophagosomes following Res treatment with IR in SU-2 cells. N, nucleus. (Scale bar = 5 μm for left column and 0.5 μm for right column). Mean ± SD. n = 3. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared with the control group. **$p < 0.01$ compared with the IR alone group.
damage was determined by measuring the tail length of the comet with a microscope at various times after IR. Compared with cells in the untreated group, remarkable DNA damage was observed in the other groups, but no obvious difference in DNA damage was found between the IR alone and co-treated groups immediately after irradiation ($p < 0.001$ and $p > 0.05$, respectively). However, DNA damage in the co-treated group was more severe than that in the IR alone group at 3 and 12 h after irradiation as reflected by the percentage of cells with the comet’s tail ($p < 0.001$ and $p < 0.05$, respectively) (Fig. 5A and B). These results suggested that co-treated GSCs repaired DNA damage less efficiently than GSCs in other groups. Therefore, Res prevented the repair of radiation-induced DNA damage in GSCs.

3.6. Res increases autophagy and apoptosis in nude mouse model

To investigate whether the results in vitro cells can also be found in vivo animal models, we performed xenograft experiments using a xenograft modal of SU-2 cells into nude mice. After cell inoculation for 14 days, the mice were injected with saline, Res, or treated with IR as mentioned above. In 16 days of treatment, the tumors in the four groups increased in a time-dependent manner. IR or Res displayed no apparent toxicity, as determined by body weight. However, the RTVs in the co-treated and Res-treated groups were significantly smaller than those in the IR alone or control group ($p < 0.01$ and $p < 0.05$, respectively). The RTVs of the co-treated, Res alone, IR alone, and control groups were 5.58 ± 0.8, 7.04 ± 0.7, 8.92 ± 0.9, and 8.41 ± 0.6, respectively (Fig. 6A). These findings demonstrated that combined treatment with Res and IR resulted in a substantial growth delay and subsequent inhibition of the growth rate of SU-2 cell xenografts.

To assess the apoptosis effect of Res combined with radiotherapy, the level of apoptosis in GSC xenograft tumors treated as described above was measured. The levels of Bcl-2 in the Res and co-treated groups significantly decreased compared with those in the control group, and the administration of Res and IR significantly reduced the expression of Bcl-2 compared with the IR alone group ($p < 0.001$) (Fig. 6B and C). Thus, the administration of Res and IR increased apoptosis in vivo.

The quantification of the percentage of LC3-I and LC3-II expression in each of the treatment groups showed a remarkable increase in the conversion of LC3-I to LC3-II in SU-2 cell xenografts.
that received combined treatment \((p < 0.01)\) (Fig. 6B and C). TEM was used to further confirm the induction of autophagy. We determined that co-treatment increased the number of double-membrane autophagosomes, as indicated by arrows in tumor sections obtained from our in vivo tumor study (Fig. 6D).

Collectively, these data suggested that the combination of Res and IR increased apoptosis and autophagy in our nude mouse model.

4. Discussion

Studies on GSCs have been conducted for many years, and GSCs have been found to contribute to the recurrence and resistance to irradiation, a major therapeutic modality for the treatment of malignant gliomas \((34–37)\). Radioresistance is a major obstacle to successful treatment in glioma radiotherapy. The use of radiosensitizer holds the promise of overcoming radioresistance and improving treatment outcomes. Therefore, novel and more effective radiosensitizers are of critical need to meet this challenge. The overall purpose of the present study was to evaluate the effect of Res on radiosensitivity in GSCs and further understand the molecular mechanisms of the synergistic effect of Res and radiotherapy in the control of gliomas. We revealed for the first time that the significant radiosensitization ability of Res in both in vitro GSCs and in vivo nude mouse model was attributed to its synergistic antitumor effects, including inhibition of proliferation, induction of autophagy, promotion of apoptosis, and prevention of DNA repair.

In our study, as evidenced by the clonogenic assay, Res could effectively inhibit GSC proliferation. The combination of Res and IR significantly reduced the colony formation capacity of GSCs (Fig. 1),
which was consistent with previously published reports (38). These results strongly suggested a radiosensitizing role of Res in GSCs. Emerging evidence suggested that CSCs are able to self-renew and are refractory to cancer treatment (26,39). Indeed, the amount and size of the newly formed neurospheres and the level of neural stem cell marker CD133 decreased in the co-treatment group (Fig. 2), which indicated that combined Res and IR deregulated the self-renewal of GSCs and reduced the stemness of GSCs. Programmed cell death is the predominant mechanism of death induced by exposure to radiation. Recent studies have indicated that the development of radioresistance is partly attributed to the inhibition of apoptosis (18,40). Anti-apoptotic Bcl-2 family members are shown to be frequently upregulated in various human tumors, and they are also overexpressed in radioreistant phenotypes of various cells (23,41). The radiosensitization efficiency of Res was substantiated by performing cellular apoptosis assays. Apoptosis in the co-treated group was more significant than that in the other groups at 48 h after IR as indicated by both annexin V-FITC/PI and Hoechst 33258 staining (Fig. 4). These results were consistent with the findings of another study that involved prostate cancer cells (42). In addition, autophagy, which is a catabolic process involving the degradation of cytoplasmic proteins and organelles through the lysosomal machinery, is also frequently activated in tumor cells following anticancer therapies, such as chemotherapeutic drugs or irradiation, and autophagy can either contribute to cell death or represent a mechanism of resistance to these treatments (6,21,28,43). Moreover, a complex interplay between cell death and survival, including necrosis, apoptosis, and autophagy, may govern tumor metastasis and subsequent carcinogenesis (44). Thus, autophagy clearly played a dual role in cancer cells depending on cell type, context, function time, or stage of tumor development. Although the exact role of autophagy underlying glioma radioresistance has remained elusive, previous studies in our laboratory have implicated that the induction of autophagy by curcumin or rapamycin promotes differentiation of GSCs and their radiosensitivity (21,27). Consistent with these observations, our data showed that the basal activity of autophagy in the untreated GSCs was very low, and radiation alone did not lead to a significant change in LC3-II level and Beclin-1. However, autophagy activity was remarkably induced in groups following the administration of Res and IR
These data suggested that the combination of Res and radiation increased radiosensitization of GSCs partly because of the induction of autophagy. De et al. recently proposed that a combinational approach of low-dose calorie restriction and Res can activate autophagy by inhibiting the mTOR pathway and/or by activating the AMPK pathways to protect 26-month-old rat hearts from doxorubicin-induced toxicity (45). However, the signaling pathway involved in the induction of autophagy by Res and IR as a mechanism of radiosensitization in GSCs needs further exploration, and autophagy inhibitors or knockdown of autophagy should be adopted to confirm whether the increased radiosensitivity in GSCs results from autophagy induction. Therefore, inhibition of the anti-apoptotic functions of these Bcl-2 family members and inhibition of the phosphatidylinositol 3-kinase (PI3K)/Akt/mTOR signaling pathway regulating autophagy (46) should be investigated as a strategy for increasing radiosensitivity of GSCs.

Moreover, DNA damage is one of the focal targets of radiation in the cellular system, and the DNA repair pathway is one of the main causes of resistance to radiation (34). Changes in the levels of DNA repair proteins have been correlated with resistance to anti-cancer strategies (47). We and others have reported that inhibition of the DNA-dependent protein kinase catalytic subunit radiosensitizes GSCs by inducing autophagy and apoptosis (45,49). Many studies have also shown that natural compounds can inhibit DNA repair machinery when coupled with radiation, thereby acting as radiosensitizing agents (50,51). In the present study, DNA damage in the co-treated group was more severe than that in the IR alone group at 3 and 12 h after irradiation as reflected by the tail length of the comet (Fig. 5). Therefore, Res impaired the repair of radiation-induced DNA damage in GSCs, which was reasonable to understand its mechanism of radiosensitization.

Most encouragingly, the synergistic effect of Res and IR in increasing radiosensitization of GSCs was applicable to animal models in vivo. Combined treatment with Res and IR resulted in a substantial growth delay and subsequent inhibition of the growth rate of tumor in the mice bearing GSCs. Similarly, in the mice bearing GSC xenograft tumors treated with Res and IR, LC3-II significantly increased and Bcl-2 remarkably decreased. In addition, double-membrane autophagosomes, as detected by TEM, significantly increased in the mice bearing GSCs treated with Res and IR (Fig. 6). These results suggest that Res coupled apoptosis with autophagy in IR mice bearing GSCs.

Our data demonstrated that the administration of Res and IR was a potential therapeutic strategy for treating GBM. The radiosensitization effect of Res was supported by impairing the capacity for DNA repair in the early stage, inhibition of proliferation and stemness, induction of autophagy, and promotion of apoptosis in vitro and in vivo. Collectively, these results demonstrated that Res was a potential radiation sensitizer for malignant glioma treatment and warrants further investigation.

**Conflict of interest**

The authors declare that there are no conflicts of interest.

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