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Full paper

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

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A B S T R A C T

Glioblastoma 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.

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1. Introduction

Glioblastoma (GBM) is the most frequent and highly aggressive primary brain tumor in adults, characterized by increased proliferation, resistance to radiotherapy and chemotherapy, and invasion into surrounding normal brain tissues (1,2). The current standard treatments for malignant gliomas include surgical resection, radiation therapy, and chemotherapy (3,4). Unfortunately, the prognosis of patients with GBM remains extremely poor because of the radioresistance of malignant gliomas despite recent technical advances in radiotherapy (5). However, a growing body of evidence suggested that only a subpopulation of malignant glioma cells, named glioma stem cells (GSCs), are resistant to radiation therapy, and this property contributes to the poor treatment outcomes associated with these tumors (6–8). Consequently, this cell population may be targeted to develop new molecular therapies against GBM.

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-

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dependent manner, and autophagy and apoptosis play important roles in Res-induced death of U251 cells (20). Furthermore, we have demonstrated that autophagy plays an essential role in the regulation of self-renewal, differentiation, tumorigenic potential, and radiosensitization of GSCs (21). However, the potential radiosensitization effect of Res and its mechanisms in GSCs both in vitro and in vivo have not been clearly revealed. Given the important role of GSCs in radioresistance, the potential radiosensitization effect of Res on GSCs and its possible mechanisms should be investigated to determine its therapeutic value in preventing or treating this disease.

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% CO_2 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 A&D LD) at a dose rate of 198 MV/min. Clonogenic survival assay was performed as described previously (23). The number of colonies with at least 50 cells was counted. Surviving fraction (SF) was calculated as: mean colony count/cells inoculated × 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 OsO_4, 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 mm^3 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 ($V = 0.5 \times a \times b^2$) was calculated. The individual relative tumor volume (RTV) was calculated as follows: $RTV = Vt/V_0$, where Vt is the volume on each day, and V_0 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.

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