

Radiosensitizing effect of 5-aminolevulinic acid-induced protoporphyrin IX in glioma cells *in vitro*

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Abstract. 5-Aminolevulinic acid (ALA) is a prodrug used in photodynamic therapy and fluorescence-guided resection of malignant gliomas due to its high cellular uptake in tumours. Porphyrin compounds act not only as photosensitizers but also as radiosensitizers. In the present study, the possible use of 5-ALA as a radiosensitizer for malignant gliomas was examined *in vitro*. Rat glioma cell lines (9L, C6) were pre-treated with 5-ALA and exposed to ionizing irradiation. The radiosensitizing effect of 5-ALA was evaluated by colony-forming assay. Intracellular reactive oxygen species (ROS) produced by 5-ALA and irradiation were evaluated by confocal laser scanning microscopy. Pre-treatment with 5-ALA enhanced the radiosensitivity of 9L cells to single-dose ionizing irradiation compared with controls (D_0 value, 4.35 ± 0.20 and 4.84 ± 0.23 Gy, respectively, $P \leq 0.05$). Exposure to multi-dose ionizing irradiation revealed high radiosensitivity in both 9L and C6 cells pre-treated with 5-ALA compared to controls. Production of intracellular ROS increased in 9L cells pre-treated with 5-ALA after ionizing irradiation compared to control cells. Thus, 5-ALA functions as a specific radiosensitizer for malignant gliomas. Intracellular 5-ALA-induced PpIX plays an important role in the production of ROS and the radiosensitizing effect under ionizing irradiation conditions.

Introduction

Malignant gliomas are highly aggressive tumours characterized by a recurrence rate of virtually 100%, despite several treat-

ment options, including aggressive surgical resection, radiation therapy and chemotherapy (1,2). Photodynamic therapy (PDT) is an effective treatment method for several kinds of neoplasms and is based on the use of different photosensitizers (3-6). In general, the effectiveness of a photosensitizer depends on two important properties, namely the production of cytotoxic oxygen and fluorescence excited by laser irradiation, which are important for PDT and fluorescence-guided resection in neurosurgery (7-9). Recent studies revealed the radiosensitizing effects of porphyrin compounds such as Photofrin and haematoporphyrin derivatives (HpD) (10-12). However, to date, few studies have provided evidence of the effect of 5-aminolevulinic acid (ALA)-induced porphyrin IX (PpIX) as radiosensitizer. Furthermore, the radiosensitizing effect of 5-ALA-induced PpIX is controversial (13-15).

In the present study, the radiosensitizing effect of 5-ALA-induced PpIX was investigated in glioma cells *in vitro*. The cytotoxic effect of combined treatment with 5-ALA and ionizing irradiation was evaluated by colony-forming assays in glioma cells. The formation of reactive oxygen species (ROS) in response to this combined treatment was assessed in glioma cells using confocal laser scanning microscopy. The effectiveness of 5-ALA-induced PpIX as a radiosensitizer is also discussed.

Materials and methods

Chemicals. 5-ALA was purchased from Cosmobio K.K. (Tokyo, Japan) and dissolved in fresh culture medium at a final concentration of 1 mM for the *in vitro* study. 2',7'-Dichlorofluorescein diacetate (DCFDA) was purchased from Sigma-Aldrich Japan K.K. (Tokyo, Japan). DCFDA was dissolved in fresh culture medium at a final concentration of 10 μ M. Other materials were of the highest grade available.

Cell culture. Two different rat glioma cell lines (9L gliosarcoma and C6 glioma) were used in this study. 9L gliosarcoma and C6 glioma cells were cultured for several days in RPMI-1640 and Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum at 37°C before use, respectively. Both cell

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lines were maintained in a humidified incubator with 5% CO₂ at 37°C. Cells were passaged in the exponential growing phase using a 0.05% trypsin solution containing 0.5 mM EDTA.

Evaluation of the response of cells to single-dose ionizing irradiation. The 9L gliosarcoma and C6 glioma cells were seeded at a density of 100 cells (0 and 2 Gy), 200 cells (4 Gy), 400 cells (6 and 8 Gy) and 600 cells (10 Gy) per 60-mm culture dish, based on preliminary measurements to determine the optimal cell concentration for each irradiation dose. The culture dishes were kept in a light-protected humidified chamber to avoid activation of 5-ALA-induced PpIX. Cells in the 5-ALA group were incubated in complete medium containing 1 mM 5-ALA for 4 h and then washed with PBS. After the medium was replaced with 3 ml of fresh culture medium, cells were irradiated by a γ -irradiator (Gammacell 40 Extractor; Nordion International, Inc., Kanata, Ontario, Canada) at a dose-rate of 0.8 Gy/min. During ionizing irradiation, culture dishes were kept in a dark container at room temperature. Control cells were treated with the same procedure as those in the 5-ALA group but without exposure to 5-ALA. The response of the cells to ionizing irradiation was evaluated by standard colony-forming assay. After 12 days of irradiation treatment, cells were fixed and stained using a Diff Quick Staining kit (Sysmex Co., Kobe, Japan). Three or four culture dishes were prepared for each dose point and the experiment was performed three times, independently. Only colonies containing ≥ 50 cells were scored. Plating efficiency was determined for unirradiated controls treated in the same way and maintained under the same conditions. The surviving fraction was then calculated. Radiation survival curves were plotted as logarithms of the surviving fraction against radiation dose and analyzed using the single-hit multi-target model (16). The 37% survival dose (D_0 value) was calculated from the slope of the exponential cell-killing line.

Evaluation of the response of cells to multi-dose ionizing irradiation. The 9L gliosarcoma and C6 glioma cells were seeded at a density of 100 cells (0 Gy) and 400 cells (total 8 Gy), respectively, per 60-mm culture dish. Cells in the 5-ALA group were incubated with complete medium containing 1 mM 5-ALA for 4 h and then washed with PBS. After the medium was replaced with 3 ml of fresh culture medium, cells were exposed to ionizing irradiation (2 Gy/day, 0.8 Gy/min), and incubated in the dark. The next day, cells were treated with 5-ALA and exposed to ionizing irradiation again. This procedure was performed for 4 consecutive days. Thus, cells were exposed to ionizing irradiation for a total of 8 Gy (2 Gy/day, 4 days). Cells in the control group were treated with the same procedure as those in the 5-ALA group but without exposure to 5-ALA. After 8 days of irradiation, cells were fixed and stained. The surviving fraction was then calculated as previously described.

Subcellular localization of 5-ALA-induced PpIX in glioma cells in vitro. Cells were seeded into 35-mm glass-bottom dishes (Asahi Techno Glass Co., Tokyo, Japan) in fresh medium containing 1 mM 5-ALA and incubated in the dark at 37°C for 4 h. Cells were then washed with PBS and examined by confocal laser scanning microscopy (LMS5 Pascal; Zeiss)

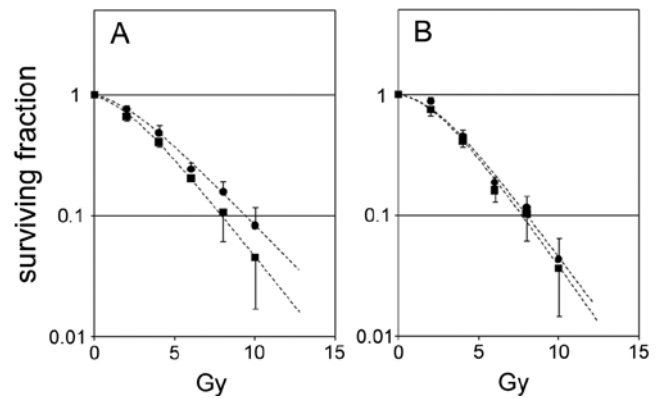


Figure 1. Radiation dose-response curves of (A) 9L gliosarcoma and (B) C6 glioma cells. The symbols ■ and ● represent cells pre-treated with 1 mM of 5-ALA for 4 h before ionizing irradiation and cells pre-treated without 5-ALA, respectively. Results represent the means of 3 independent assays, each performed in 3 or 4 culture dishes at each dose point. Error bars indicate standard deviation (SD).

with excitation/emission wavelength set at 488/560 nm with a long-pass filter, respectively.

Detection of intracellular reactive oxygen species (ROS) in glioma cells in vitro. Intracellular ROS production was detected using the oxidant-sensitive fluorescent probe DCFD with a confocal laser scanning microscope (17,18). Cells were seeded into 35-mm glass-bottomed dishes in fresh medium containing 1 mM 5-ALA and incubated in the dark at 37°C for 4 h. After the incubation, cells were washed with PBS twice, incubated with 10 μ M DCFD for 10 min, washed with PBS and exposed to 10 Gy of ionizing irradiation. DCF fluorescence (excitation, 488 nm; emission, 505- to 530-nm band-pass filter) and 5-ALA-induced PpIX (excitation, 488 nm; emission, 560-nm long-pass filter) was imaged on a confocal laser scanning microscope (LMS5 Pascal, Zeiss). All procedures were performed in the dark.

Statistical analyses. The data are presented as mean \pm SD and were analyzed with Fisher's protected least significant difference. Significance was defined as $P \leq 0.05$.

Results

5-ALA-induced PpIX-mediated response of cells to single-dose ionizing irradiation. Fig. 1 shows the enhanced effect of 5-ALA-induced PpIX on the radiosensitivity of exponentially growing 9L and C6 cells to single-dose ionizing irradiation. The error bars on the survival curves indicate standard deviation calculated from 3 independent experiments. Based on irradiation dose-response curves corresponding to the multi-target single-hit model, 9L cells treated with 5-ALA before single-dose ionizing irradiation showed higher radiosensitivity (D_0 value, 4.35 ± 0.20 Gy) than control cells (D_0 value, 4.84 ± 0.23 Gy) ($P \leq 0.05$) (Figs. 1A and 2). However, there were no significant differences in the response of C6 cells to single-dose ionizing irradiation between the 5-ALA treated group and the control (D_0 value, 4.26 ± 0.10 and 4.51 ± 0.17 Gy, respectively) ($P = 0.096$) (Fig. 1B).

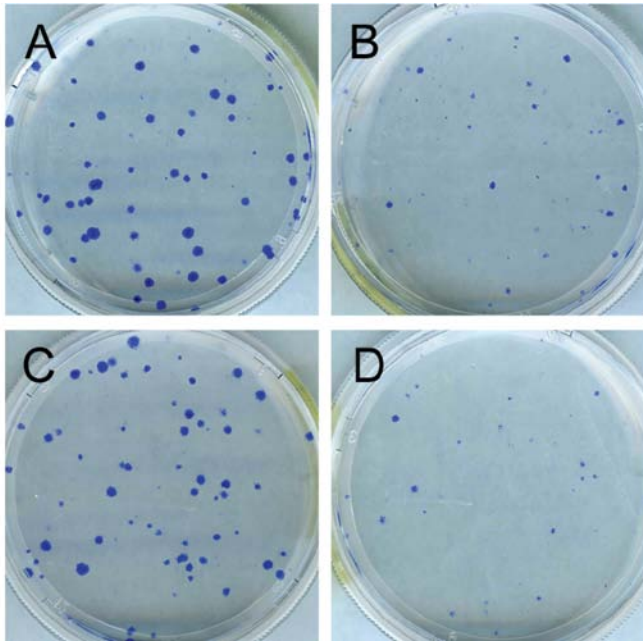


Figure 2. Representative culture plate of standard colony forming assay in 9L gliosarcoma cells. (A, B) Cells without 5-ALA before ionizing irradiation; (C, D) cells pre-treated with 1 mM 5-ALA before ionizing irradiation. (A, C) and (B, D) represent control (0 Gy) and ionizing irradiation (8 Gy), respectively. Colonies were fixed and stained using Diff Quick Staining kit.

Cumulative radiosensitizing effect of 5-ALA to multi-dose ionizing irradiation. To evaluate the cumulative radiosensitizing effect of 5-ALA, glioma cells were exposed to multi-dose ionizing irradiation and the number of colonies were counted. Cells treated with 5-ALA in each irradiation revealed higher radiosensitivity in both 9L and C6 cells, compared to control ($P \leq 0.05$) (Fig. 3).

Subcellular localization of 5-ALA-induced PpIX in glioma cells. Fig. 4 shows that 5-ALA-induced PpIX was located mainly in the cytoplasm in both 9L and C6 cells. However, the fluorescence intensity of PpIX in 9L cells was markedly higher than that of C6 cells (Fig. 4).

ROS production in glioma cells after ionizing irradiation. Intracellular ROS generated by ionizing radiation in 9L cells were visualized by DCF fluorescence using the oxidant-sensitive probe DCFD (Fig. 5). Our imaging condition of confocal laser scanning microscopy, as previously described, did not detect an interaction between 5-ALA-induced PpIX and DCF fluorescence (Fig. 5B and E). DCF fluorescence was concentrated in the cytoplasm of glioma cells after ionizing irradiation. Pre-treatment of cells with 5-ALA before irradiation caused an increase in DCF fluorescence (Fig. 5J). Interestingly, DCF fluorescence generated by ionizing irradiation predominantly coincided with 5-ALA-induced PpIX in the cytoplasm of 9L cells (Fig. 5L). In addition, PpIX fluorescence decreased slightly after ionizing irradiation (Fig. 5I), compared to controls without ionizing irradiation (Fig. 5A). Although C6 cells were subjected to the same imaging methods, DCF fluorescence in these cells was very faint (data not shown).

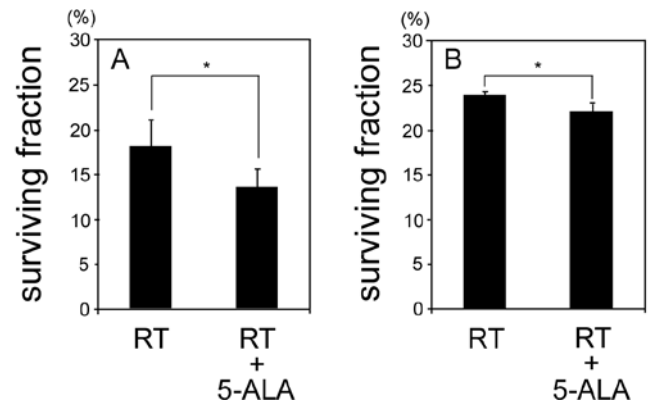


Figure 3. Cumulative radiosensitizing effect of 5-ALA induced by multi-dose ionizing irradiation. (A) 9L gliosarcoma, (B) C6 glioma. Irradiation (RT) + 5-ALA. Cells were pre-treated with 5-ALA and exposed to ionizing irradiation for 4 consecutive days. RT, irradiation only. The total dose of ionizing irradiation was 8 Gy in each group. Bars, SD, * $P \leq 0.05$.

Discussion

5-ALA-induced PpIX as a specific radiosensitizer in glioma cells. Enhancement of tumour responses to ionizing irradiation by 5-ALA-induced PpIX in glioma cells was confirmed using a standard colony-forming assay *in vitro*. The present data demonstrate that 5-ALA-induced PpIX affects the radiosensitivity of glioma cells with variation according to the cell line irradiated. Porphyrin compounds, in particular HpD and Photofrin, have been found to act as efficient radiosensitizing agents for tumours *in vitro* and *in vivo* (10,11,13,14). Recent studies reported the efficacy of gadolinium texaphyrin (Gd-Tex), which is a pentadentate aromatic metalloporphyrin, identified as a tumour-selective sensitizer for ionizing irradiation in clinical studies (19,20). Previous reports showed low intracellular concentrations and a weak radiosensitising effect of 5-ALA-induced PpIX in response to single-dose ionizing irradiation compared to HpD and Photofrin (12,14,15). However, the present results suggest that multi-dose ionizing irradiation enhances the radiosensitizing effect of 5-ALA-induced PpIX, and thus inhibits cell proliferation in glioma cells *in vitro* (Fig. 3). These data indicate that 5-ALA-induced PpIX has a radiosensitizing effect in gliomas and is appropriate for multi-dose ionizing irradiation.

Potential mechanism of the radiosensitizing effect of 5-ALA-induced PpIX. The mechanism underlying the radiosensitizing effect of 5-ALA-induced PpIX has not been elucidated to date. In the present study, differences in the fluorescence intensity of intracellular 5-ALA-induced PpIX were observed between 9L and C6 cells (Fig. 4). In 9L cells, high intracellular PpIX concentrations correlated with enhanced inhibition of cell proliferation by ionizing irradiation compared to C6 cells. These results suggest that the radiosensitizing effect depends on the intracellular concentration of PpIX, which concurs with other reports (14). We confirmed the production and localization of intracellular ROS induced by ionizing irradiation in glioma cells pre-treated with 5-ALA using confocal laser scanning microscopy *in vitro*. Irradiation of cells in combination with 5-ALA treatment resulted in the enhanced production of

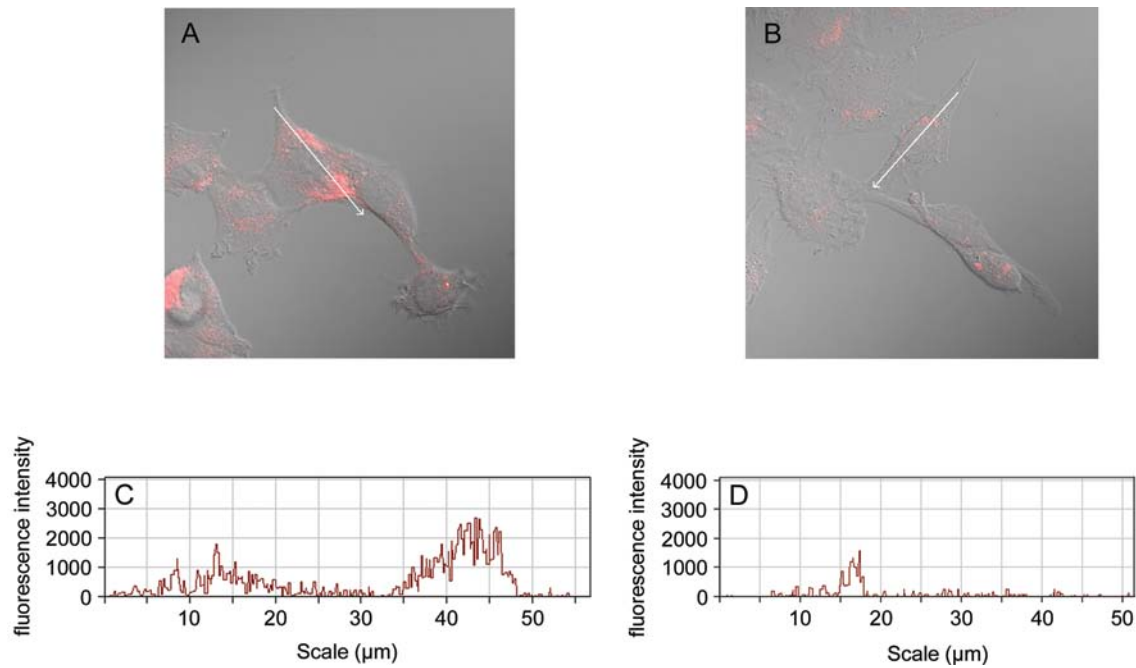


Figure 4. Visualization and quantification of intracellular 5-ALA-induced PpIX in 9L gliosarcoma (A, C) and C6 glioma cells (B, D) *in vitro*. Micrographs show the fluorescence image of intracellular 5-ALA-induced PpIX merged with the DIC image (A, B). The fluorescence intensity of the micrographs of intracellular 5-ALA-induced PpIX was plotted.

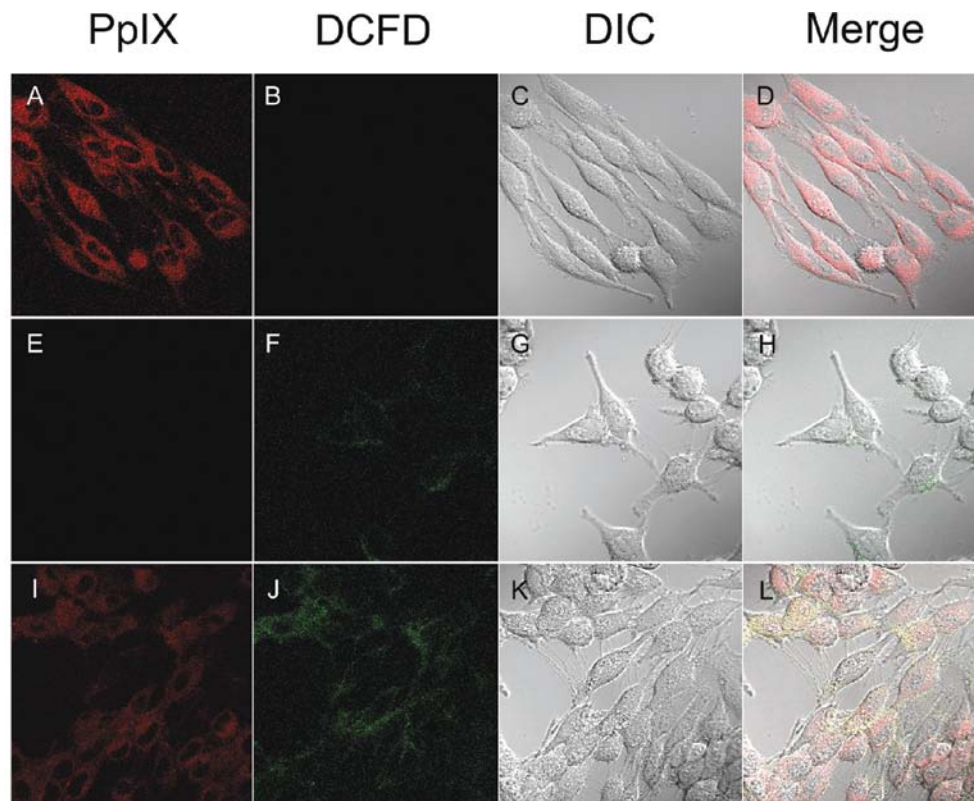


Figure 5. Visualization of intracellular 5-ALA-induced PpIX and ROS induced by ionizing irradiation in 9L gliosarcoma cells *in vitro*. (A-D) Cells treated with 1 mM 5-ALA, no ionizing irradiation. (E-H) Cells pre-treated with 10 μM DCFD and then exposed to ionizing irradiation (10 Gy). (I-L) Cells pre-treated with 1 mM 5-ALA and 10 μM DCFD and then exposed to ionizing irradiation (10 Gy). (A, E and I) Fluorescence image of intracellular 5-ALA-induced PpIX. (B, F and J) DCF fluorescence image. (C, G and K) DIC image. (D, H and L) Merged image corresponding to the fluorescence of intracellular 5-ALA-induced PpIX, DCF fluorescence and the DIC image. The intracellular localization of 5-ALA-induced PpIX and ROS was analyzed using confocal laser scanning microscopy (fluorescence and DIC images). DCF fluorescence was mainly accumulated in the cytoplasm of glioma cells after ionizing irradiation (F, J). Note that DCF fluorescence increased when cells were treated with 5-ALA and then irradiated (J). Interestingly, DCF fluorescence was predominantly co-localized with 5-ALA-induced PpIX (L) and the fluorescence of intracellular PpIX was reduced after ionizing irradiation (I), compared to control (A).

intracellular ROS compared to irradiation without 5-ALA in 9L cells. To the best of our knowledge, this is the first report to show the production and intracellular localization of ROS in response to ionizing irradiation concurrent with 5-ALA treatment. In general, the damaging effect of ionizing radiation on living cells is mainly attributed to the effects of ROS, including superoxide, hydroxyl radical and hydrogen peroxide, generated by the decomposition of water. Secondary radicals formed by the interaction of hydroxyl radicals with organic molecules are thought to play an important role in cellular damage (21,22). ROS can damage critical cellular components such as DNA, proteins and lipids, and eventually lead to physical and chemical damage to tissues (23). Some studies speculate that Photofrin may enhance the radiolytic activity by reacting with cytotoxic, activated molecules such as hydroxyl radicals, which are known to be generated as a result of the primary interaction of ionizing radiation with water (24). In the present study, intracellular ROS produced by ionizing irradiation co-localized with 5-ALA-induced PpIX in 9L cells (Fig. 5L). In addition, ionizing irradiation reduced the fluorescence of intracellular PpIX (Fig. 5A and B). There are two possible explanations for this phenomenon: i) intracellular PpIX may produce ROS in response to ionizing irradiation, which would affect its own formation or ii) ROS produced by intracellular PpIX and ionizing irradiation may damage the cell membrane and induce leakage of intracellular PpIX to the extracellular space. Taken together, the present results suggest that intracellular 5-ALA-induced PpIX plays an important role in the production of ROS and the radiosensitizing effect under ionizing irradiation conditions.

We evaluated the radiosensitizing effect of 5-ALA-induced PpIX in different glioma cell lines *in vitro*. The damaging effect of ionizing irradiation depends on the amount of intracellular ROS produced by intracellular 5-ALA-induced PpIX in glioma cells. Although the radiosensitizing effect of 5-ALA-induced PpIX is weak, multi-dose ionizing irradiation enhances the radiosensitizing effect of 5-ALA-induced PpIX in glioma cells.

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