

5-Aminolevulinic acid-induced protoporphyrin IX with multi-dose ionizing irradiation enhances host antitumor response and strongly inhibits tumor growth in experimental glioma *in vivo*

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Abstract. Ionizing irradiation is a well-established therapeutic modality for malignant gliomas. Due to its high cellular uptake, 5-aminolevulinic acid (ALA) is used for fluorescence-guided resection of malignant gliomas. We have previously shown that 5-ALA sensitizes glioma cells to irradiation *in vitro*. The aim of the present study was to assess whether 5-ALA acts as a radiosensitizer in experimental glioma *in vivo*. Rats were subcutaneously injected with 9L gliosarcoma cells and administered 5-ALA. The accumulation of 5-ALA-induced protoporphyrin IX was confirmed by high-performance liquid chromatography (HPLC) analysis. Subcutaneous (s.c.) tumors were subsequently irradiated with 2 Gy/day for five consecutive days. In the experimental glioma model, high-performance liquid chromatography analysis revealed a high level of accumulation of 5-ALA-induced protoporphyrin IX in s.c. tumors 3 h after 5-ALA administration. Multi-dose ionizing irradiation induced greater inhibition of tumor growth in rats that were administered 5-ALA than in the non-5-ALA-treated animals. Immunohistochemical analysis of the s.c. tumors revealed that numerous ionized calcium-binding adapter molecule 1 (Iba1)-positive macrophages gathered at the surface of and within the s.c. tumors following multi-dose ionizing irradiation in combination with 5-ALA administration. By contrast, the s.c. tumors in the control group scarcely showed aggregation of Iba1-positive macrophages. These results suggested that multi-dose ionizing irradiation with 5-ALA induced not only a direct cytotoxic effect but also enhanced the host antitumor

immune response and thus caused high inhibition of tumor growth in experimental glioma.

Introduction

Photodynamic therapy (PDT) with photosensitizers, including hematoporphyrin derivatives (HpD), photofrin and 5-aminolevulinic acid (ALA)-induced protoporphyrin IX (PpIX), has been used in the treatment of several types of cancer (1-3). The effectiveness of photosensitizers depends on two important properties: The production of cytotoxic oxygen and fluorescence upon excitation by laser irradiation (4-6). In particular, 5-ALA has high affinity for malignant gliomas, and thus, it is commonly used for fluorescence-guided tumor resection in clinical neurosurgery (4). Although several porphyrin compounds, including HpD and photofrin, have been shown to act as radiosensitizers, the radiosensitizing activity of 5-ALA-induced PpIX remains controversial (7-11). We previously demonstrated that although the ability of 5-ALA-induced PpIX to radiosensitize glioma cell lines *in vitro* was weak, multi-dose ionizing irradiation may be used to enhance this radiosensitizing effect (12). The aim of the present study was to further evaluate the radiosensitizing effects of 5-ALA-induced PpIX in combination with multi-dose ionizing irradiation by assaying this activity in a rat subcutaneous (s.c.) glioma model. The potency of 5-ALA-induced PpIX as a radiosensitizer for cancer therapy was also discussed.

Materials and methods

Chemicals. 5-ALA was purchased from Cosmo Bio K.K. (Tokyo, Japan) and was dissolved in phosphate-buffered saline (PBS; WAKO Pure Chemical Industries, Ltd, Osaka, Japan) at a concentration of 100 mg/ml. The pH of the solution was adjusted to 6.0-6.3 with 10 N sodium hydroxide (NaOH; WAKO Pure Chemical Industries, Ltd) and checked using pH indicator paper. The solution was used within 10 min of preparation. 5-ALA was intravenously administered to rats via the tail vein at a dose of 100 mg/kg body weight.

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Brain tumor cell lines and animals. All the following experiments were performed in accordance with an animal protocol approved by the Institutional Animal Care and Use Committee (University of Occupational and Environmental Health, Kitakyushu, Japan). The 9L gliosarcoma cell line, which was generated from inbred Fischer rats, has been widely used as a syngeneic rat model for experimental gliomas. Originally produced by *N*-methyl-nitrosourea mutagenesis in Fischer rats by Benda *et al* (13) and Schmidek *et al* (14) at Massachusetts General Hospital, the tumor was obtained by Barker at the University of California, cloned and designated 9L gliosarcoma due to the dual appearance of a glioblastoma and a sarcoma. 9L gliosarcoma cells rapidly proliferate under *in vitro* and *in vivo* conditions, and are the most widely used cells in experimental rat glioma, for example in brain and subcutaneous tumor models (6,15). 9L gliosarcoma cells were obtained from Dr Tsutomu Tokuyama at the Hamamatsu University School of Medicine (Hamamatsu, Japan) and cultured for several days in RPMI-1640 medium (WAKO Pure Chemical Industries, Ltd) supplemented with 10% fetal calf serum (FCS; Nichirei Biosciences Inc., Tokyo, Japan) before use. Syngeneic male Fischer 344 rats (8 weeks of age; mean body weight, 167 g) were purchased from SLC, Inc. (Hamamatsu, Japan). A total of 32 rats were used for the present study. Animals were inoculated with 9L gliosarcoma cells, as previously described (3,6). Briefly, these cells (1×10^6) were implanted into the dorsal skin of the Fisher 344 rats, and thereby, a rat s.c. tumor model was prepared for the following experiments. All animals were kept at a constant room temperature of 24°C under a 12-h light/dark cycle (7 am to 7 pm) in the laboratory animal center of the University of Occupational and Environmental Health. In addition, all animals received sufficient food, which was sterilized and certified for experimental animals (MF; Oriental Yeast Co., Ltd, Tokyo, Japan), according to the animals requirements.

Evaluation of the accumulation of 5-ALA-induced PpIX in the rat s.c. tumor model. Firstly, the accumulation of 5-ALA-induced PpIX was confirmed in the established rat s.c. tumor model using high-performance liquid chromatography (HPLC) analysis and fluorescence observation. Once the tumors had grown to ~1 cm in diameter, the rats were given an intravenous (i.v.) injection of 5-ALA (100 mg/kg body weight) into the tail vein. Three hours later, the rats were anesthetized and tumor specimens without the dorsal skin cover were removed and immediately snap-frozen in liquid nitrogen, then stored at -80°C in the dark for HPLC analysis. According to the previously described method of HPLC analysis of porphyrin metabolites (16,17), tumor specimens (1-mm diameter) were treated with 200 μ l of 0.1 M NaOH and homogenized on ice with a Powermasher II (Assist, Tokyo, Japan). An aliquot (10 μ l) of the NaOH-treated samples was withdrawn and used for a protein concentration assay (Quick Start™ Bradford Dye Reagent, Bio-Rad Laboratories, Inc., Hercules, CA, USA), while the remaining 50 μ l of cellular proteins were denatured by the addition of 150 μ l *N,N*-dimethylformamide/isopropanol solution (100:1, v/v; Nacalai Tesque, Inc., Kyoto, Japan). After overnight storage in the dark, the prepared samples were subjected to HPLC analysis performed as previously described (2,17) with certain modifications. Briefly, porphyrins were separated using a Prominence HPLC system (Shimadzu, Kyoto, Japan) equipped with a

reversed-phase C18 column (CAPCELL PAK, C18, SG300, 5 μ m, 4.6 mmx250 mm; Shiseido, Tokyo, Japan) maintained at 40°C. The elution solvents used were solvent A (1 M ammonium acetate including 12.5% acetonitrile, pH 5.2) and solvent B (50 mm ammonium acetate including 80% acetonitrile, pH 5.2; Kanto Chemical Co., Inc., Tokyo, Japan). Elution was performed with solvent A for 5 min and subsequently with a linear gradient of solvent B (0-100%) for 25 min, followed by elution with solvent B for 10 min. The elution flow was maintained at a constant rate through the use of a fluorospectrometer (excitation at a wavelength of 404 nm, detection at a wavelength of 624 nm; F7000; Hitachi, Tokyo, Japan). The porphyrin concentrations in the samples were estimated using calibration curves obtained with standard porphyrins (Protoporphyrin IX; Sigma-Aldrich, St. Louis, MO, USA).

In addition, under anesthesia, the rat dorsal skin covering the inoculated tumors was evaginated and the s.c. tumors were observed underneath the skin at 3 h post-intravenous administration of 100 mg/kg 5-ALA. Bright-field images of the s.c. tumors were captured using a digital camera (D90, Nikon Corporation, Tokyo, Japan) with a long-pass filter and an external halogen lamp as the light source (C-FID, Nikon). Subsequently, the same s.c. tumors were illuminated with ultraviolet light (410-nm light-emitting diode illuminator, SBI Pharma CO., Ltd., Tokyo, Japan), and tumor images were captured using a digital camera with a long-pass filter.

Evaluation of the *in vivo* radiosensitizing effects of 5-ALA with multi-dose ionizing irradiation in a rat s.c. tumor model. Syngeneic Fischer 344 rats were inoculated with 9L gliosarcoma cells, as previously described (3,6). A previous study demonstrated that the growth of s.c. 9L tumors in syngeneic Fischer rats was inhibited at >10 Gy with single-dose ionizing irradiation (18). Other previous studies reported that for PDT, the dose of 5-ALA used for a single i.v. administration was 100-500 mg/kg for rodents (6,19-21). Thus, in the present study, the maximal dose of ionizing irradiation and 5-ALA administration used were 10 Gy and 500 mg/kg. A previous study by our group confirmed that multi-dose ionizing irradiation with 5-ALA inhibited tumor proliferation *in vitro* (12). However, multiple i.v. injection via the rat tail vein was technically difficult due to injury and obstruction of vessels. According to preliminary experiments by our group, up to 5 i.v. injections were possible. Therefore, the optimal ionizing irradiation schedule was determined to be 2 Gy and 5-ALA administration (100 mg/kg) per day, for five consecutive days. Once the s.c. tumors had grown to a diameter of 6-8 mm, the animals were randomly divided into four groups and treated as follows: Control group, no further treatment (n=5); 5-ALA administration (n=5); ionizing irradiation (RT) (n=7); and ionizing irradiation with 5-ALA administration (5-ALA + RT) (n=7). In the 5-ALA administration group, the animals received 5-ALA (100 mg/kg) alone for five consecutive days without ionizing irradiation. In the ionizing irradiation with 5-ALA administration group, the animals were anesthetized 3 h post-5-ALA administration and the s.c. tumors were irradiated with 2 Gy in the dark using an X-ray irradiator (MBR-1520R; HITACHI Engineering & Service Co., Ltd., Japan) at a rate of 0.65 Gy/min. The animals were completely covered with an X-ray shield sheet, except for the tumor regions, to avoid excessive exposure

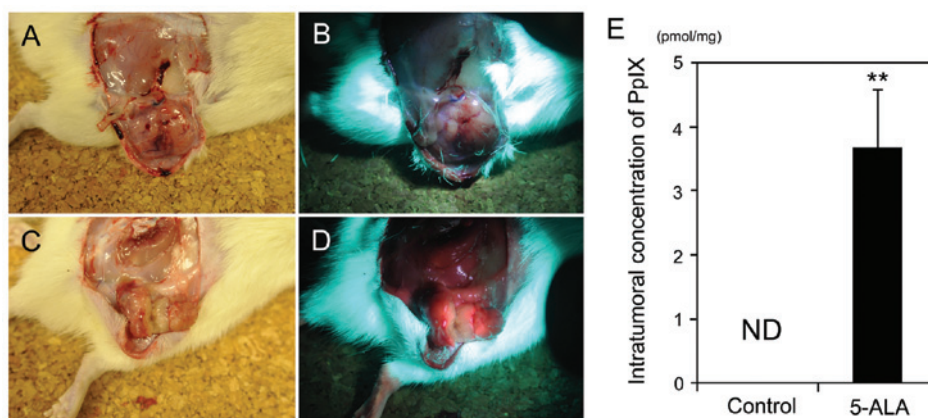


Figure 1. Representative images of a rat s.c. tumors following surgical exposure and HPLC analysis of the intratumoral concentration of PpIX. (A and C) Bright-field image showing the rat s.c. tumor. (B and D) Fluorescence image showing the tumor illuminated by light of wavelength 410 nm. (D) The s.c. tumor strongly expressed red fluorescence, originating from porphyrin, 3 h after the intravenous administration of 5-ALA, as compared with (B) the control tumor. (E) HPLC analysis showed a high accumulation of 5-ALA-induced PpIX in the tumor after 5-ALA administration (E). Data are presented as the mean (pmol/mg-protein) \pm the standard error of the mean. ** $P < 0.01$ compared with the control; ND, not detected. 5-ALA, 5-aminolevulinic acid; PpIX, protoporphyrin IX. HPLC, high-performance liquid chromatography; s.c., subcutaneous.

of the rest of the body to the ionizing irradiation. This procedure was performed for five consecutive days, resulting in exposure to a total of 10 Gy (2 Gy/day, 5 days). In the ionizing irradiation group, the s.c. tumors were irradiated in an identical manner, without the administration of 5-ALA. To avoid photochemical effects, all animals were kept in the dark for 12 h after 5-ALA administration. Thereafter, direct exposure of the animals to light was avoided. Tumor growth was assessed every 2 days until 16 days post-treatment. Tumor volumes were calculated using the formula $V = a^2b/2$, where a and b are the shortest and longest perpendicular diameters, respectively (22). Sixteen days after treatment, the animals were sacrificed under deep anesthesia using equithesin (0.4 ml/100g), which was composed of chloral hydrate, magnesium sulfate, ethanol and propylene glycol, all purchased from WAKO Pure Chemical Industries Ltd, as well as pentobarbital sodium salt (Tokyo Chemical Industry Co., Ltd, Tokyo, Japan). All tumor specimens were immediately removed with the dorsal skin cover and fixed in 20% formaldehyde/PBS for further pathological examination.

Pathological examination. Following fixation all tumor specimens were cut at the center of the tumor in the direction of the longest axis. Sections were stained with hematoxylin and eosin and ionized calcium-binding adapter molecule 1 (Iba1) for macrophage detection, and all staining processes were outsourced to Pathology Institute Corp, Toyama, Japan. Briefly, after a water bath pretreatment (40 min, 95°C), deparaffinized sections were washed with KN buffer (KN-09002; Pathology Institute Corp., Toyama, Japan). The sections were incubated with goat polyclonal anti-Iba1 (1:3,000, ab107159; Abcam, Cambridge, UK) for 30 min, followed by a further wash with KN buffer. Subsequently, the sections were incubated with Simple Stain™ MAX-PO (G) (H1301; Nichirei Bioscience Inc.) for 30 min. Following a final wash with KN buffer, color development was performed with diaminobenzidine (DAB; DAKO, Glostrup, Denmark) for 10 min and the sections were counter-stained with hematoxylin (WAKO Pure Chemical industries, Ltd). Thereafter, all tumor specimens were evaluated in our laboratory. Based on a previous study (23), microdensitometry

for quantitative evaluation of Iba1 immunohistochemistry was performed on digital microphotographs (Colorio EP-705A; Seiko Epson Corp., Suwa, Japan) using the public domain software ImageJ 1.46r (National Institutes of Health, Bethesda, MD, USA), with certain modifications of the original method. In brief, all Iba1-stained tumor specimens were scanned using a color scanner (Colorio EP-705A) and then photographed. To perform quantification of the immunohistochemical DAB color signal of Iba1, image data of all samples were transferred to ImageJ and converted to an 8-bit grey-scale image. The free-hand tool was used to delineate the whole-tumor section as the region of interest (ROI) for in each tumor specimen, and the mean gray value (MGV) within the ROI was plotted on a graph. The representative value was defined as the average MGV of all the five tumor specimens in the control group and the relative intensity of the MGV of the other groups was calculated according to the representative value.

Statistical analyses. Data are presented as the mean \pm the standard error of the mean. Statistical analyses were performed using StatView 5.0 software (SAS Institute, Cary, NC, USA). The mean tumor volume was analyzed using unpaired two-sample t -tests, and the relative intensity of MGV of Iba1 was calculated using Fisher's exact probability tests. Statistical significance was defined as $P < 0.05$.

Results

Intratumoral accumulation of 5-ALA-induced PpIX in the rat s.c. tumor model. In the established rat s.c. tumor model, tumors demonstrated strong fluorescence 3 h after the intravenous administration of 5-ALA, compared with that of the control tumors without 5-ALA administration (Fig. 1). Furthermore, HPLC analysis revealed that the amount of 5-ALA-induced PpIX in tumors was 3.66 ± 0.91 pmol/mg-protein 3 h after the intravenous administration of 5-ALA, which was significantly higher than that of the control ($P < 0.01$) (Fig. 1E). By contrast, 5-ALA-induced PpIX was not detected in the control tumors without 5-ALA administration (< 0.1 pmol/mg-protein).

Table I. Effect of 5-ALA and multi-dose RT on tumor growth.

Group	Day 0	Day 8	Day 10	Day 12	Day 14	Day 16
Control (n=5)	142.3±13.8	1191.5±128.8	1793.2±199.2	2296.8±233.3	2799.0±281.9	3747.1±254.1
ALA (n=5)	143.9±17.4	865.4±107.2	1403.0±224.3	1842.6±218.8	2276.3±192.8	2869.4±267.9
RT (n=7)	149.9±14.9	683.3±99.4	1059.0±101.7	1485.8±133.4	1736.9±162.9	2035.2±245.9
ALA + RT (n=7)	146.0±12.9	385.3±59.7	495.6±91.1	667.6±119.7	819.0±135.5	863.1±147.4
P-value ^a	0.8457	0.0246	0.0014	0.0007	0.001	0.0015

Values are expressed as the mean tumor volume (mm³) ± the standard error of the mean. Brackets indicate number of sample. ^aRT vs ALA + RT (unpaired t-test). 5-ALA, 5-aminolevulinic acid; RT, ionizing irradiation.

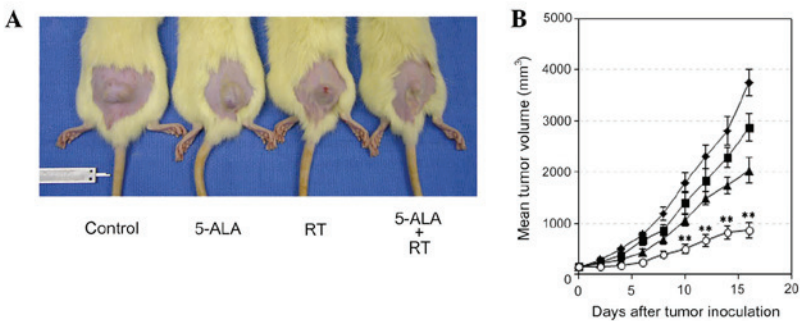


Figure 2. Representative image of rat subcutaneous (s.c.) tumors at day 16 after treatment. (A) The hair covering the skin was removed. (B) Growth curves of the s.c. tumors. Once the s.c. tumors had grown to a diameter of 6–8 mm, the animals were randomly divided into four groups and treated as follows: (◆) Control (n=5); (■) 5-ALA (n=5); (▲) multi-dose RT (n=7); and (○) multi-dose RT with 5-ALA (n=7). The control group did not receive any treatment. Rats in the 5-ALA group received 5-ALA (100 mg/kg) via the tail vein for five consecutive days. Rats in the multi-dose ionizing irradiation group were exposed to 2 Gy/day of RT for five consecutive days (total 10 Gy). Rats in the multi-dose irradiation with 5-ALA group were exposed to RT (2 Gy/day) 3 h after the intravenous administration of 5-ALA (100 mg/kg) for five consecutive days. Each point represents the mean, and the bars represent the standard error of the mean; **P<0.01 versus the multi-dose RT group (▲). s.c., subcutaneous; 5-ALA, 5-aminolevulinic acid; RT, ionizing irradiation.

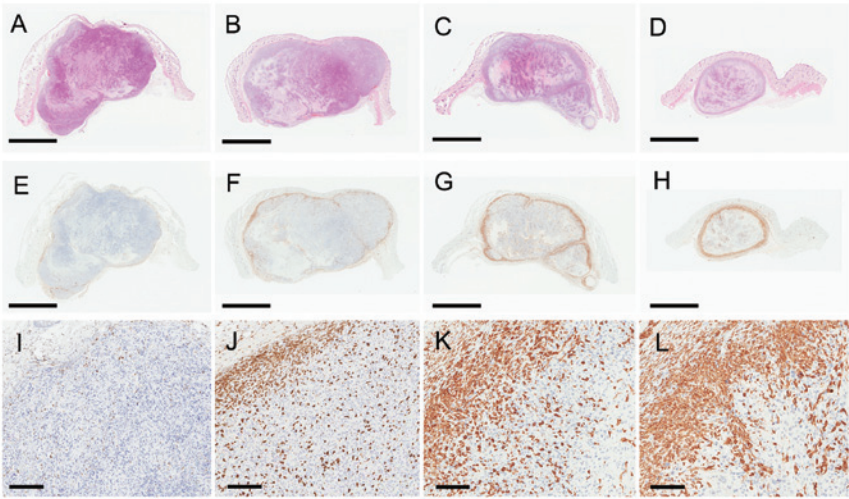


Figure 3. Pathology results of sc tumors at day 16 after treatment in the (A, E, I) control, (B, F, J) 5-ALA, (C, G, K) multi-dose RT, and (D, H, L) multi-dose RT with 5-ALA groups. (A–D) (HE) staining and (E–L) Iba1 staining. (E–H) The Iba1-stained sections correspond to the HE-stained sections in each group. (I–L) Magnified views of the Iba1 staining of the surface of the s.c. tumors in each group. (A–D) The s.c. tumors showed coagulative necrosis in each group. (H, L) Iba1-positive macrophages gathered at the surface of, and within, the s.c. tumors in the multi-dose ionizing irradiation with 5-ALA group. In contrast, (E, I) Iba1-positive macrophages were scarcely observed in the control group. Scale bar: (A–H) 5 mm and (I–L) 200 μm. 5-ALA, 5-aminolevulinic acid; RT, ionizing irradiation; HE, hematoxylin and eosin; Iba1, ionized calcium-binding adapter molecule 1.

Radiosensitizing effect of 5-ALA with multi-dose ionizing irradiation in vivo. The subcutaneously inoculated 9L gliosarcomas grew at a near exponential rate (Fig. 2, Table I). On the first day

(day 0) of tumor treatment, there was no difference in tumor size between the RT and 5-ALA + RT groups (P=0.8457, Table I). Treatment with multi-dose ionizing irradiation and 5-ALA

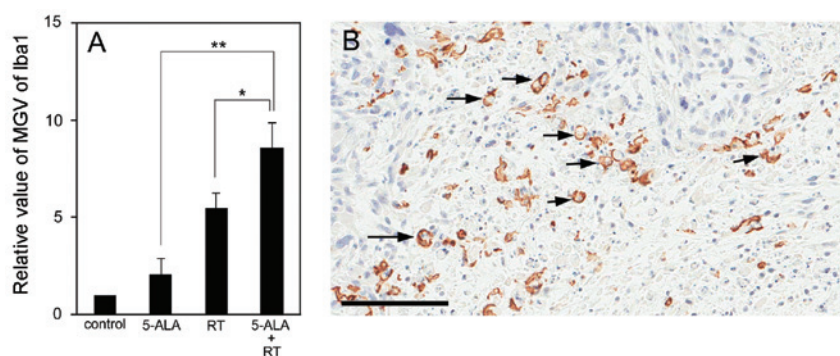


Figure 4. Microdensitometric analysis. (A) Relative intensity of the MGVs of Iba1. The MGV of the multi-dose ionizing irradiation with 5-ALA group was significantly higher than that of the other groups. (B) Representative pathological findings of the boundary zone between the coagulation necrosis and the surviving tumor cells in the subcutaneous tumor in the multi-dose ionizing irradiation with 5-ALA group (Iba1 staining). Iba1-positive macrophages gathered in the boundary zone between the coagulation necrosis and the surviving tumor cells. Note that numerous Iba1-positive macrophages showed phagocytic features (arrows). Values are expressed as the mean \pm the standard error of the mean. * $P < 0.05$ and ** $P < 0.01$ compared with the control (A). Scale bar: 120 μ m. MGV, mean gray value; 5-ALA, 5-aminolevulinic acid; RT, ionizing irradiation; Iba1, ionized calcium-binding adapter molecule 1.

significantly inhibited tumor growth, compared with that of tumors treated with irradiation alone ($P < 0.01$, day 10) (Fig. 2 and Table I). On day 16, the mean inhibition of tumor growth in the 5-ALA + RT group was 42.5% of that in the RT group (Fig. 2A and Table I). Of note, tumor growth was inhibited by 5-ALA administration without treatment with ionizing irradiation as compared with that of the control group ($P = 0.0448$ at day 16).

Histological evaluation of s.c. 9L gliosarcomas after multi-dose ionizing irradiation. The tumor specimens showed coagulative necrosis within the tumors, with variance among the groups (Fig. 3A-D). Of note, the Iba1-positive macrophages also showed a certain variance among the groups (Fig. 3E-L). The control group showed a very low accumulation of Iba1-positive macrophages in the s.c. tumors (Fig. 3E and I). In the 5-ALA group, Iba1-positive macrophages mainly gathered at the surface of the s.c. tumors, only slightly invading the tumors (Fig. 3E and J). Similarly, numerous Iba1-positive macrophages gathered at the surface of and within the s.c. tumors following treatment with ionizing irradiation (Fig. 3G and K) and in particular following treatment with ionizing irradiation in combination with 5-ALA (Fig. 3H and L). Microdensitometric analysis showed that significantly more Iba1-positive macrophages gathered in the s.c. tumors of the ionizing irradiation + 5-ALA group compared with those of the other groups ($P < 0.05$ vs. RT; $P < 0.01$ vs. 5-ALA) (Fig. 4A). Analysis of the distribution of the Iba1-positive macrophages within the tumors revealed that the Iba1-positive macrophages did not gather within the zone of coagulation necrosis, but primarily gathered in the boundary zones between the coagulation necrosis and the surviving tumor cells. In particular, a number of Iba1-positive macrophages displayed features of the phagocytic process (Fig. 4B).

Discussion

The results of the present study confirmed that 5-ALA-induced PpIX has a radiosensitizing effect in experimental glioma. It was determined that tumor growth was significantly inhibited in 5-ALA-treated irradiated rats compared with rats exposed to multi-dose ionizing irradiation in the absence of 5-ALA. This was in agreement with a previous *in vitro* study by our group (12). To the best of our knowledge, the present study was

the first to show the radiosensitizing effects of 5-ALA-induced PpIX and to confirm the immunological effects in experimental glioma *in vivo*.

The mechanism underlying the radiosensitizing effects of porphyrin compounds remains elusive. Using confocal laser scanning microscopy, it was previously demonstrated that intracellular 5-ALA-induced PpIX has an important role in the production of reactive oxygen species and in radiosensitization (12). This radiosensitizing effect was found to depend on the intracellular concentrations of porphyrin compounds, including HpD and photofrin, which were low following 5-ALA administration (6). Therefore, high intracellular concentrations of HpD and photofrin may have a strong radiosensitizing effect with single-dose ionizing irradiation, comparable with that of 5-ALA-induced PpIX *in vitro* and *in vivo* (8-10, 24). In certain cell lines, however, 5-ALA-induced PpIX was shown to have a low sensitizing effect with single-dose ionizing irradiation (12,25). In addition, another study indicated that ionizing irradiation increased, rather than inhibited, ALA-induced synthesis of PpIX in human colon adenocarcinoma cells *in vitro* (9). In the present study, 5-ALA-induced PpIX was found to significantly enhance tumor sensitivity to multi-dose ionizing irradiation. Therefore, repeated administration of 5-ALA, combined with ionizing irradiation, may enhance the radiosensitizing effect of 5-ALA-induced PpIX, thereby strongly inhibiting tumor growth in experimental glioma.

In addition, the immunological response to 5-ALA-induced PpIX and multi-dose ionizing irradiation was measured by performing immunohistochemical staining with Iba1 for macrophages. The combination of 5-ALA-induced PpIX with multi-dose ionizing irradiation resulted in a strong aggregation of Iba1-positive macrophages at the surface of and within the s.c. tumors. Iba1 expression is typically upregulated in activated macrophages/microglia, which exhibit a distinct morphology with an amoeboid shape and short processes (26,27). Macrophages may be broadly divided into the following two groups: *i*) Classically activated M1-type macrophages that typically participate in the coordinated response to immunogenic antigens, primarily through the production of proinflammatory mediators, including interleukin (IL)-1 β , IL-12 and tumor necrosis factor- α (TNF- α), and have an overall enhanced ability to phagocytose

pathogenic material (28,29); and *ii*) alternatively activated M2-type macrophages that do not secrete proinflammatory mediators such as IL-1B and TNF- α (30) and are believed to exert immunomodulation primarily through the secretion of the potent immunosuppressive cytokines IL-10 and TGF- β and have a decreased phagocytic capacity (31,32). In the present study, numerous Iba1-positive macrophages gathered at the surface of and within the s.c. tumors following 5-ALA administration of multi-dose ionizing irradiation. In particular, these Iba1-positive macrophages with phagocytic features primarily gathered in the boundary zones between the coagulation necrosis and the surviving tumor cells. By contrast, although coagulative necrotic changes were revealed within the s.c. tumors in all the groups, Iba1-positive macrophages scarcely gathered at the surface of the s.c. tumors in the control group. Thus, these Iba1-positive macrophages did not gather at the surface of the s.c. tumors merely for the removal of the coagulative necrotic tissues within the tumors. Therefore, it is hypothesized that 5-ALA-induced PpIX with multi-dose ionizing irradiation treatment induced not only a direct cytotoxic effect, but additionally a long-lasting immunological effect following the ionizing irradiation, including the induction of tumor cytotoxic M1-type macrophages, and consequently caused a strong inhibition of tumor growth.

A previous study reported that photodynamic therapy with 5-ALA led to an increase in tumor-infiltrating mononuclear cells and the activation of intraperitoneal macrophages in Lewis lung carcinoma (33). Of note, the present study found that repeated administration of 5-ALA alone inhibited tumor growth. While it was not possible to completely exclude all photodynamic effects, the results indicated that even when exposure to room light was limited and indirect, 5-ALA alone enhanced the host antitumor immune response. It has been suggested that the mechanisms of these immunological effects with response to 5-ALA in cancer therapy shall be investigated in greater detail in future studies (34). A recent clinical study showed that 5-ALA administered orally once a day over a 12-week period reduced both fasting and postprandial glucose levels in type-2 diabetes mellitus without adverse effects (35). Patients with malignant brain tumors frequently receive fractionated radiotherapy following surgical resection. Various radiotherapy modalities, including stereotactic radiotherapy (SRT), stereotactic radiosurgery (SRS) and intensity-modulated radiotherapy, precisely control the intensity of ionizing irradiation, thereby avoiding or reducing the exposure of healthy tissues and limiting the side-effects of the treatment. Thus, SRT and SRS provide high doses of irradiation to small, precise areas of intracranial lesions (36,37). 5-ALA has a high affinity for malignant brain tumors and has a number of advantages, including reduced skin phototoxicity compared with that to other photosensitizers. The present study presents preliminary data of the radiosensitizing effects of 5-ALA-induced PpIX in experimental glioma. Multi-dose ionizing irradiation with 5-ALA-induced PpIX may be a novel therapeutic strategy for malignant gliomas with possible clinical applications.

The present study demonstrates the radiosensitizing effect of 5-ALA in experimental glioma *in vivo*. Multi-dose ionizing irradiation alongside 5-ALA administration induces strong inhibition of tumor growth and enhances the antitumor immune response in experimental glioma. Although investi-

gation of the mechanisms underlying the combined effects of multi-dose irradiation and 5-ALA-induced PpIX is essential to clearly define the interactions between these components, multi-dose ionizing irradiation with 5-ALA-induced PpIX may be an appropriate treatment for patients with malignant brain tumors and should be assessed in clinical trials.

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