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Full paper

Evaluation of edaravone against radiation-induced oral mucositis in mice

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ABSTRACT

Oral mucositis induced by radiotherapy for cancers of the head and neck reduce the quality of life of patients. However, effective therapeutic agents are lacking. Symptomatic treatment involves local anesthesia and analgesia. We focused on the antioxidant effects of edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one; Radicut®). Oral mucositis was induced on the tongue tips of mice using a single dose of X-rays (20 Gy). To evaluate the protective effect of edaravone (30 and 300 mg/kg), administration was carried out 30 min before irradiation. Survival, oral mucositis score, myeloperoxidase activity, and levels of 2-Thiobarbituric acid reactive substances were measured, and all were improved compared with those of control mice. A significant difference was not found in terms of survival due to edaravone. Histopathologic findings also highlighted the beneficial features of edaravone. Edaravone reduced the production of reactive oxygen species. These findings suggest that the protective effect of edaravone against radiation-induced oral mucositis is through an antioxidant effect.

1. Introduction

Oral mucositis is a common side-effect of chemotherapy and radiotherapy. Moderate-to-severe mucositis occurs in virtually all patients who receive radiotherapy for cancers of the head and neck (1). Mucositis is a painful and debilitating side-effect of radiotherapy for cancers of the head and neck (2). Radiotherapy can cause erythematous, erosive and ulcerative mucositis, which can result in a decrease in food intake. Swallowing difficulties are associated with dehydration and weight loss. Furthermore, mucositis reduces the quality of life (QOL) of patients because communication becomes very difficult.

Local anesthetics, low-level laser irradiation, anti-ulcer agents, and anti-inflammatory agents have been used for the treatment of oral mucositis (3–6). However, the clinical response elicited by these drugs against oral mucositis is poor.

X-ray-induced cell death results from two types of actions, direct and indirect. In the first type, X-rays ionize or excite macromolecules in cells directly, leading to cell damage. In the second type, X-rays excite water molecules in the cells and produce reactive oxygen species (ROS), which damage cells. It has been found that approximately 70% of the biological damage caused by X-rays results from this indirect action (7). ROS such as the oxygen radical (O_2^-) and hydrogen peroxide (H_2O_2) are detoxified easily by antioxidant defense enzymes, whereas cytotoxic ROS, such as the hydroxyl radical (•OH), cannot be detoxified by these enzymes. During radiotherapy, the detrimental effects of ionizing radiation on biological tissues such as the skin are mediated primarily by cytotoxic ROS such as •OH, which induces excess apoptosis (8).

Edaravone is a brain-protective agent used to treat acute ischemic stroke. The mechanism of action of edaravone is based on free-radical scavenging. The initial stage of radiation damage involves formation of free radicals, and edaravone could be used to help prevent lethal damage from ionizing radiation (9).

In the present study, we used mice for the evaluation of edaravone against radiation-induced oral mucositis (RIOM). Mice have been used as an *in vivo* model for RIOM (10, 11), so it is thought that

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this model of RIOM is valid. Several studies have evaluated the effect of edaravone on radiation damage in a mouse model (9, 12). Edaravone has been reported to show a radioprotective effect if given 30 min before irradiation (9). In addition, it has been reported that edaravone can suppress X-ray-induced apoptosis in hippocampal neurons (12). However, no study has evaluated the action of edaravone against RIOM on the tips of the tongues of mice. Therefore, we aimed to evaluate the effectiveness of edaravone against RIOM in mice.

2. Materials and methods

The experimental protocol was conducted according to the guidelines set by the Ethics Review Committee for Animal Experimentation of Ehime University Medical School (Ehime, Japan).

2.1. Animals

Six-week-old ICR mice (30–40 g; Japan SLC Inc., Shizuoka, Japan) were used in all experiments. Animals were housed in a room maintained at 22 ± 2 °C under a 12-h light–dark cycle with lights on at 7:00 a.m. Mice were fed a standard rodent diet and had free access to water.

2.2. RIOM

Mice (n = 20 per group) were anesthetized (pentobarbital sodium, 50 mg/kg body weight, i.p.) and then irradiated. Mice had to be irradiated only at the tip of the tongue, so the rest of the body was shielded with a lead device (thickness, 0.5 mm). The tongue was fixed to the outer surface of the lead device using adhesive tape and irradiated with a single radiation dose of 20 Gy. Radiation was generated using a 150-kV potential (20 mA) X-ray source at a focal distance of 350 mm hardened with a 1.0-mm aluminum filtration system (MBR-1520R-3; Hitachi, Tokyo, Japan). The rate of radiation administration was 5.1 Gy/min.

2.3. Drugs

Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one; Radicut®) was provided by Mitsubishi Tanabe Pharma Corporation (Osaka, Japan). A 2-Thiobarbituric Acid Reactive Substances (TBARS) Microplate Assay kit was purchased from Funakoshi Corporation (Tokyo, Japan).

2.4. Edaravone treatment

Edaravone was dissolved in a small volume of 1 M NaOH solution. The pH was adjusted to 7 with 1 M HCL. The concentration was adjusted to 3 mg/mL or 15 mg/mL in 0.9% (physiologic) saline solution (9).

Body weight was recorded and tongues observed after irradiation every day. Edaravone was injected *via* the intraperitoneal route 30 min before irradiation. The control group was irradiated but edaravone was not administered.

2.5. Assessment of mucositis

The scoring of oral mucositis was based on a modification of the method of Sonis et al. (13). To assess the severity of oral mucositis, mice were anesthetized with isoflurane every day. The oral mucositis score was: 0 = normal; 1 = partial hyperemia, erythema and swelling; 2 = overall hyperemia, erythema and swelling; 3 = epidermolysis, hyperemia and erythema; 4 = extensive epidermolysis and bleeding; 5 = bleeding and abscesses.

2.6. Determination of myeloperoxidase (MPO) activity

MPO activity is a marker for neutrophils in inflamed tissue. MPO activity was measured in mouse tongues using a modification of the method of Chen et al. (14). After mice had been killed by cervical dislocation 12 days after irradiation, tongue samples (n = 10 per group) were removed and stored at −70 °C until required for assay. Samples were weighed and homogenized in 10 volumes of 50 mM potassium phosphate buffer (pH 6) containing 0.5% hexadecyltrimethylammonium bromide (Sigma–Aldrich Japan, Tokyo, Japan) for 1 min. After freezing and thawing homogenates thrice, they were centrifuged at 10,000 × *g* for 15 min at 4 °C. Supernatants were collected and reacted with 0.167 mg/mL *o*-dianisidine dihydrochloride (Wako Biochemicals, Osaka, Japan) and 0.0005% H_2O_2 (Wako Biochemicals) in 50 mM phosphate buffer (pH 6). MPO activity was measured using a Microplate Reader (NJ-2300; Biotec, Tokyo, Japan) at 450 nm. MPO activity was calculated by measuring the slope of absorbance calibrated using MPO standards (Wako Biochemicals) and expressed as MPO/g tongue.

2.7. Determination of TBARS levels

TBARS are present naturally in biological specimens. They include lipid hydroperoxides and aldehydes, which increase in concentration as a response to oxidative stress.

Tongue samples (n = 10 per group) were removed after mice had been killed by cervical dislocation 12 days after irradiation, and stored at −70 °C until required for assay. Samples were weighed and homogenized in 0.5–1 mL of phosphate-buffered saline (PBS) per 100 mg of tongue. Samples were centrifuged at 1500 × *g* for 10 min at 4 °C. Supernatants were collected and diluted in assay buffer.

TBARS levels were measured using the Microplate Reader (Biotec) at 540 nm. TBARS levels were calculated by measuring the slope of absorbance calibrated using TBARS standards (Funakoshi Corporation, Tokyo, Japan) and expressed as TBARS/g tongue.

2.8. Histopathologic analyses

For evaluation of edaravone, tongues were removed for histopathologic analyses after killing mice 12 days post-irradiation. Specimens were fixed in 10% neutral-buffered formalin, dehydrated and embedded in paraffin (Wako Biochemicals). Tissue sections were obtained and stained with hematoxylin & eosin (H&E) and examined under light microscopy (×200 magnification).

2.9. Immunohistochemical (IHC) detection

For evaluation of edaravone, tongues were removed for IHC analyses after killing mice 12 days post-irradiation. We used paraffin-embedded tissue sections for TUNEL staining (×400 magnification). At first, we conducted deparaffinization, hydration and protein digestion. Subsequently, we labeled the 3-terminal ends of DNA with 100 μL (or 50 μL) of TdT Reaction Solution for 10 min at 37 °C. We washed sections with PBS, and labeled them with POD-conjugated antibody. We undertook color development with 100 μL of 3,3′-Diaminobenzidine solution at room temperature for 5 min, washed with double-distilled water, and then counter-stained. These actions were followed by dehydration, cleaning, mounting and inspection under a light microscope. We counted the number of TUNEL-positive cells on the tongue tips and expressed them as a percentage.

2.10. Statistical analyses

Results are the mean and standard error of the mean, or the mean value. Data were analyzed using one-way analysis of variance

(ANOVA) followed by the Steel–Dwass test. $P < 0.05$ was considered significant.

3. Results

3.1. Progress after irradiation

We measured body weight after irradiation every other day. Irradiation of 20 Gy to the tongues of mice led to a decrease in body weight that was maximal at day-12. After day-12, body weight increased (Fig. 1). Intake of food and water also decreased, and was accompanied by weight loss.

Fig. 2 shows the dose-dependency of edaravone (30 and 300 mg/kg). The number of mice that survived decreased after day-12. A significant difference in survival was not found. Behavioral disorders were not observed in mice even at 300 mg/kg edaravone.

3.2. Pathophysiologic observations

Pathophysiologic changes in mouse tongues were evaluated by macroscopic and histologic means. Fig. 3 shows the scoring for oral mucositis. Oral mucositis was not observed for 7 days, but developed on day-8. The severity score reached a maximum value on day-12. Thereafter, the score decreased with time. The oral mucositis score for edaravone in the 300 mg/kg-administered group was significantly lower than that of the control group on day-10 and day-12. Total scores between day-0 and day-12 for edaravone at 30 and 300 mg/kg were significantly lower than those of the control group, respectively (Fig. 4).

3.3. Histopathologic aspects

Histopathologic sections of tongue samples are shown in Fig. 5A. In the control group, epidermolysis of the tongue was observed. However, in the edaravone group, the degree of epidermolysis was preserved, and infiltration of inflammatory cells decreased.

3.4. TUNEL staining

TUNEL staining was undertaken to confirm cell injury due to radiation. The control group had considerable apoptosis (23.7% TUNEL-positive cells). In contrast, the percentage of TUNEL-positive cells was decreased to 9.9% and 8.6% after treatment with 30 and 300 mg/kg of edaravone, respectively (Fig. 6).

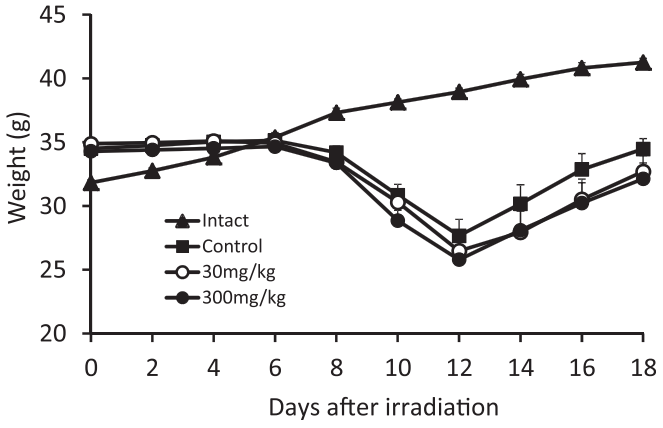

Fig. 1. Changes in body weight over time after X-ray irradiation. Each bar is the mean ± SEM.

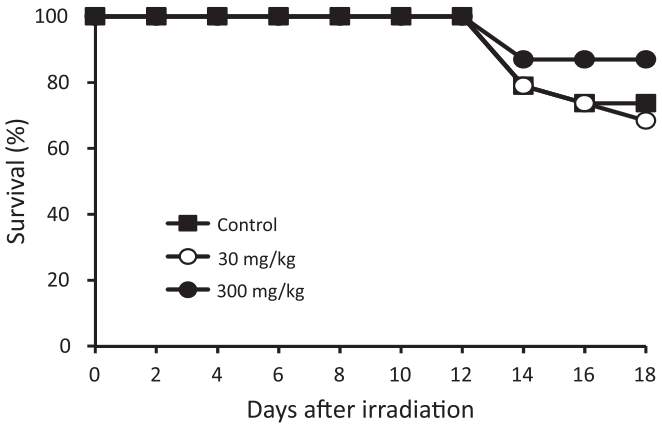

Fig. 2. Time-course of survival after X-ray irradiation.

3.5. MPO activity and TBARS levels

MPO activity and TBARS levels were measured 12 days after irradiation. MPO activity in edaravone-administered groups at 30 and 300 mg/kg was significantly lower compared with those in the control group (Fig. 7).

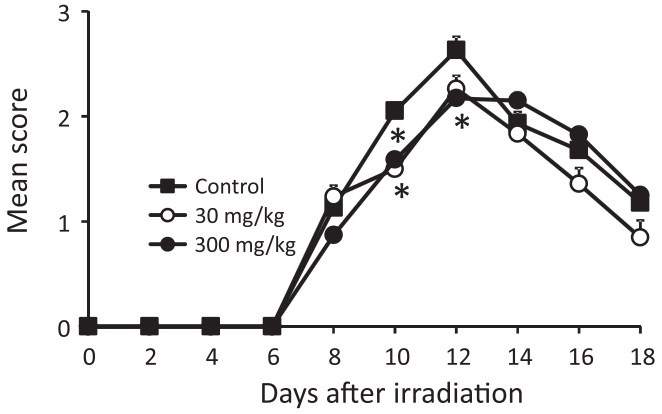

Fig. 3. Data are the mean ± SEM score of oral mucositis (n = 10). Mice tongues were irradiated with 20 Gy on day-0. *$p < 0.05$, significantly different from the control value (Steel–Dwass test).

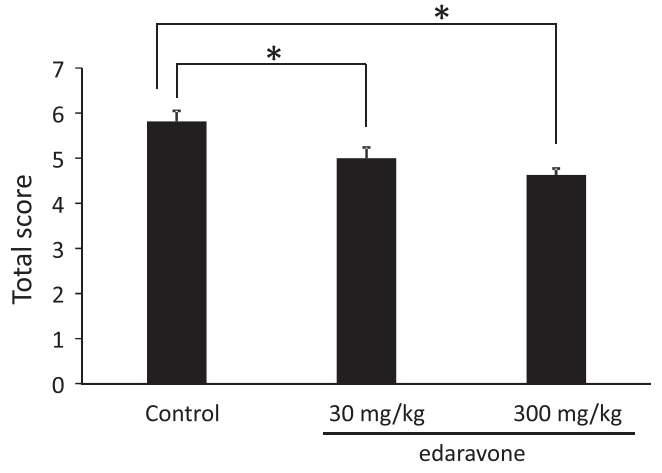

Fig. 4. Mean value of the total score of oral mucositis between day-0 and day-12 for each group. Data are the mean ± SEM (n = 10).*$p < 0.05$, significantly different from the control value (Steel–Dwass test).

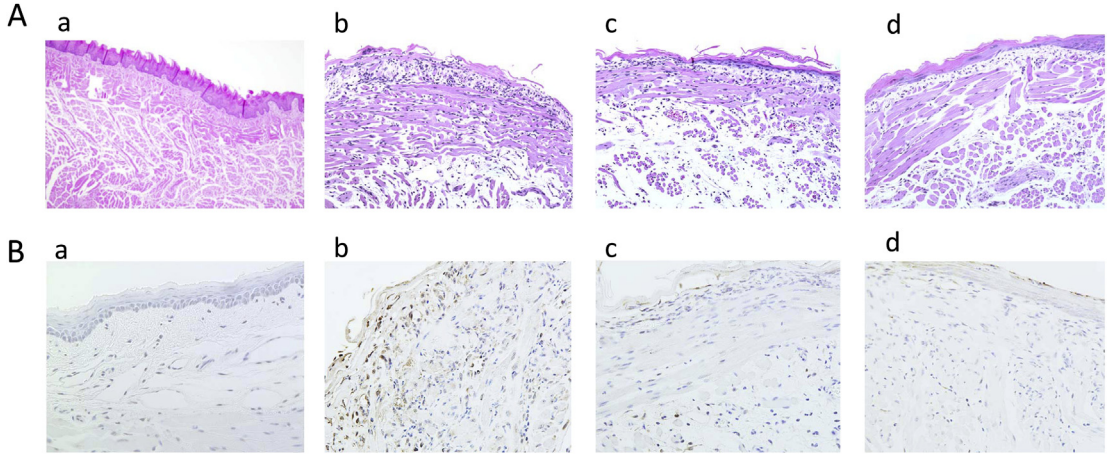

Fig. 5. Histologic photographs of tongue specimens after X-ray irradiation. Tongue specimens were fixed with 10% buffered formalin and embedded in paraffin. A: Sections (3 mm) were stained with hematoxylin and eosin (×200). a: Intact, b: control (oral mucositis), c: edaravone 30 mg/kg, d: edaravone 300 mg/kg. B: Apoptotic cells were evaluated by TUNEL staining (×400). a: Intact, b: control (oral mucositis), c: edaravone 30 mg/kg, d: edaravone 300 mg/kg.

TBARS levels in edaravone-administered groups at 30 and 300 mg/kg were lower than those of the control group, and edaravone (300 mg/kg) caused a significant decrease compared with that in the control group (Fig. 8).

4. Discussion

RIOM is a painful side-effect that can reduce QOL and lead to discontinuation of cancer therapy, prolonged hospitalization, and death. Oral mucositis is observed in almost all patients during radiotherapy, but effective agents are lacking. In the present study, the effect of edaravone against RIOM was investigated using ICR mice.

In a study by Li et al. a single dose of 30 Gy to rats was employed, and oral mucositis was observed (15). However, when we irradiated 30 Gy to mice tongues, mortality was high. Therefore, we used 20 Gy for our model of RIOM.

Food intake was decreased by the development of oral mucositis, and body weight also decreased 8 days after irradiation. Loss in body weight was maximal at day-12, but increased gradually afterwards. Radiation exposure produced oral mucositis in all mice from day-8. The oral mucositis score reached a maximum value at day-12. Onset of oral mucositis was identical to the onset of oral mucositis observed after irradiation in human subjects, so we considered our model to a suitable *in vivo* model of RIOM.

The effect of edaravone on epithelial cells was demonstrated using H&E and TUNEL staining (Fig. 5A and B). In the control group, mice tongues showed epidermolysis. Many inflammatory cells

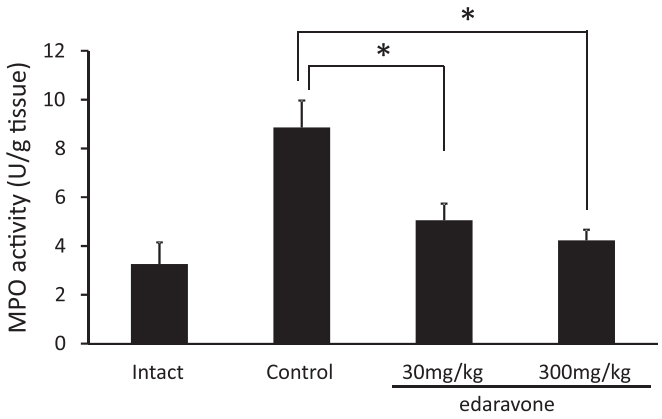

Fig. 7. Effect of edaravone on myeloperoxidase (MPO) activity (unit) in mouse tongues submitted to oral mucositis. *$p < 0.05$, significantly different from the control value (Steel–Dwass test).

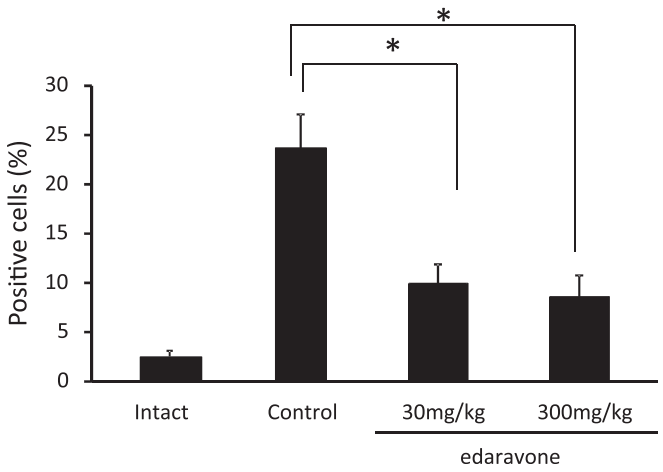

Fig. 6. Percentage apoptosis after TUNEL staining. *$p < 0.05$, significantly different from the control value (Steel–Dwass test).

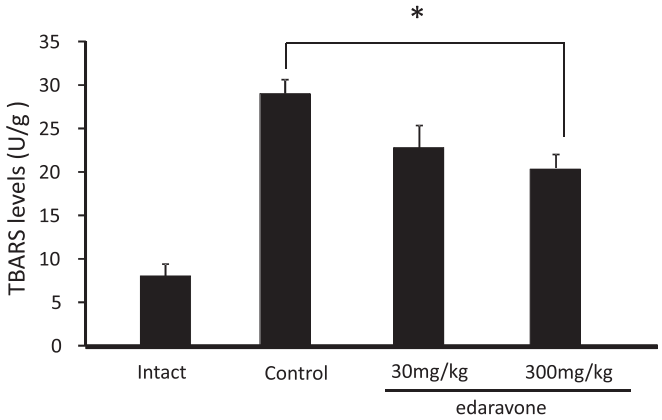

Fig. 8. Effect of edaravone on determination of 2-Thiobarbituric Acid Reactive Substances (TBARS) activity (unit) in mouse tongues submitted to oral mucositis. *$p < 0.05$, significantly different from the control value (Steel–Dwass test).

were observed in the control group but, in the edaravone-administered group, the epithelial layer was retained and fewer inflammatory cells were observed 12 days after irradiation. In addition, many TUNEL-positive cells were observed in the control group. Hence, cell injury from irradiation had occurred. Furthermore, we found that MPO activity in the edaravone-administered group was lower than that of the control group. That is, inflammation of mice tongues induced by radiation was ameliorated by edaravone administration. These results showed that edaravone was effective against RIOM.

In the present study, we focused on the antioxidant effects of edaravone. Allopurinol is used mainly to treat chemotherapy-induced mucositis, but has radical-scavenging activity against O_2^- only (16). Allopurinol inhibits release of xanthine oxidase, orotidylate decarboxylase and proteases. It also shows an antioxidant effect, consequently reducing the production of active oxygen (17). Edaravone interacts biochemically with a wide range of free radicals, donates electrons, and is transformed eventually to a stable chemical (18). X-rays excite water molecules in cells and produce ROS, which damage cells. The initial stage of radiation-induced damage involves the formation of free radicals. Edaravone is expected to be effective in preventing lethal damage from ionizing radiation (9) because it is a free-radical scavenger.

We also measured TBARS levels because they tend to be increased by radiation-induced oxidative stress. The control group had high TBARS levels (i.e., the control group experienced high oxidative stress). However, the edaravone-administered group exhibited slight increases in TBARS levels. Sasano et al. reported that a higher dose of edaravone completely eliminated intracellular generation of ROS by X-rays (19). We confirmed an antioxidant effect of edaravone against RIOM. The effects of edaravone on oral mucositis seem weak, but the antioxidant effects of edaravone are sufficient. We considered inflammation to be not only on a superficial layer but also within the tongue. Hence, evaluation of only the macroscopic score was difficult. The direct action of radiation also influences cell injury. Inflammation and tissue injury are not suppressed only by antioxidant actions. Damage to DNA can occur late after irradiation. By administering edaravone, production of the initial free radicals due to irradiation can be suppressed. Therefore, subsequent lipid peroxidation can also be suppressed. The results shown in Fig. 8 suggest that edaravone can suppress lipid peroxidation.

Several authors have reported on the antioxidant effects of edaravone. Edaravone belongs to a class of pharmacologic agents that reduce free radicals, and are associated with cholinergic dysfunction and apoptotic damage (20). Combinations of antioxidants have been shown to have synergistic anti-tumor effects *in vivo*. Combinations of antioxidants with chemotherapy and radiation have been shown to increase survival time and reduce toxicity in humans (21). It has also been shown that the tumor response to radiotherapy in patients with limited-stage prostate cancer is not inhibited by concomitant naturopathic and nutritional supplements based on the magnitude of the prostate-specific antigen (PSA) response, the velocity of the PSA nadir, and the duration of PSA normalization (22). These observations made us suspect that edaravone does not influence the anti-tumor effect of radiation.

With regard to the points mentioned above, our results suggest that the antioxidant effects of edaravone against RIOM are important. Such effects warrant further investigation.

Conflicts of interest

The authors indicated no potential conflicts of interest.

Acknowledgments

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