Edaravone protects human peripheral blood lymphocytes from γ -irradiation-induced apoptosis and DNA damage

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Abstract Radiation-induced cellular injury is attributed primarily to the harmful effects of free radicals, which play a key role in irradiation-induced apoptosis. In this study, we investigated the radioprotective efficacy of edaravone, a licensed clinical drug and a powerful free radical scavenger that has been tested against γ -irradiation-induced cellular damage in cultured human peripheral blood lymphocytes in studies of various diseases. Edaravone was pre-incubated with lymphocytes for 2 h prior to γ -irradiation. It was found that pretreatment with edaravone increased cell viability and inhibited generation of γ -radiation-induced reactive oxygen species (ROS) in lymphocytes exposed to 3 Gy γ -radiation. In addition, γ -radiation decreased antioxidant enzymatic activity, such as superoxide dismutase and glutathione peroxidase, as well as the level of reduced glutathione. Conversely, treatment with 100 μ M edaravone prior to irradiation improved antioxidant enzyme activity and increased reduced glutathione levels in irradiated lymphocytes. Importantly, we also report that edaravone reduced γ -irradiation-induced apoptosis through downregulation of Bax, upregulation of Bcl-2, and consequent reduction of the Bax:Bcl-2 ratio. The current study shows edaravone to be an effective radioprotector against γ -irradiation-induced cellular damage in lymphocytes *in vitro*. Finally, edaravone pretreatment significantly reduced DNA

damage in γ -irradiated lymphocytes, as measured by comet assay (% tail DNA, tail length, tail moment, and olive tail moment) ($p < 0.05$). Thus, the current study indicates that edaravone offers protection from radiation-induced cytogenetic alterations.

Keywords Immune depletion · Edaravone · γ -irradiation · Apoptosis · Lymphocytes

Introduction

γ -radiation has been used in curative and palliative treatments for cancerous solid tumors for several decades (Ando et al. 2014). Multiple lines of evidence have shown that γ -radiation exposure can induce a variety of cellular and sub-cellular damage in many living organisms at sub-lethal doses, including genomic instability, mutagenesis, and cell death (Garrison and Uyeki 1988). During radiotherapy, γ -irradiation interacts with biological systems to produce free radicals, such as hydroxyl radicals ($\bullet$ OH), that are formed from the radiolysis of surrounding water molecules and successively attack DNA (Khan et al. 2013). Importantly, γ -radiation can be absorbed directly by DNA, thereby leading to ionization of both nucleobases and sugars in a mechanism described as the direct effect of the generation of single and tandem DNA damage (Rezaee et al. 2013). In order to control the flux of reactive oxygen species (ROS) and protect them from free radical action, living cells have developed their own defense system, the antioxidant system, which includes enzymatic and non-enzymatic components (Sharma and Halder 2006). Since ROS overload induced by γ -radiation might exceed the cellular antioxidant capacity, molecules with direct free radical scavenging properties are especially promising as radioprotectors (Karbownik and Reiter 2000). It has been proven that antioxidants protect or reduce γ -radiation-

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induced chromosome damage, and micronuclei and primary DNA damage in human peripheral blood lymphocytes in both *in vitro* and *in vivo* investigations (Ghosh et al. 2012).

As a potent antioxidant, edaravone strongly scavenges free radicals, thereby offering protection against oxidative stress and neuronal apoptosis (Higashi et al. 2006). It has been used clinically to reduce neuronal damage resulting from cerebral ischemic stroke in Japan and China (Nakamura et al. 2008). In addition, edaravone has displayed its protective effects in a variety of brain diseases, including traumatic brain injury (Ohta et al. 2013), Alzheimer's disease (Yan et al. 2012), and Parkinson's disease (Yuan et al. 2008). Previous research has shown that edaravone plays an important role as a direct and indirect free radical scavenger by neutralizing toxic ROS and reactive nitrogen species (RNS) (Roh et al. 2011). Importantly, edaravone has been found to protect DNA from oxidative stress (Shimizu et al. 2009). These mechanisms require the presence of edaravone at the time of radiation in cells and tissues. The aim of the present study was to investigate the modulating effect of edaravone on γ -radiation-induced apoptosis and DNA damage in human peripheral blood lymphocytes.

Materials and methods

Isolated blood lymphocyte culture

Human peripheral blood samples were collected in sterile vacutainer tubes (Becton, Dickinson and Company, USA) containing lithium heparin as anticoagulant median cubital vein of nonsmoking healthy individuals (22–25 years old). Lymphocytes were isolated from blood using Ficoll–Histopaque density gradients with slight modification as described previously (Borek 2004). Briefly, after being diluted in a 1:1 ratio with phosphate buffered saline (PBS), blood samples were layered onto Histopaque with a ratio of blood+PBS:Histopaque maintained at 4:3 followed by centrifugation at 1,300 rpm for 35 min at room temperature. The lymphocyte layer was collected and maintained in RPMI-1640 media. The number of viable cells was counted by a hemocytometer.

Irradiation procedure

The different concentrations of edaravone were added to isolated lymphocytes 2 h prior to irradiation and maintained at 37 °C. Samples were irradiated at 3 Gy (dose rate 50 cGy min⁻¹) using a cobalt teletherapy Unit located at Yantai Yuhuangding Hospital. The samples were kept at 37 °C for 1 h soon after irradiation to facilitate repair mechanisms. The samples were then transported to the laboratory in an ice bath. Isolated lymphocytes were divided into three groups; in each group, six samples were processed.

Sham control: normal lymphocytes

Radiation control: γ -irradiated lymphocytes (3 Gy)

Edaravone+ γ -radiation: edaravone (100 μ M) plus irradiated (3 Gy) lymphocytes

Intracellular reactive oxygen species measurement

Intracellular ROS was determined by 2',7'-dichlorofluorescein diacetate (DCFH-DA) as previously described (Sheng et al. 2009). In a 24-well dish after irradiation, 2×10^5 cells from each group were seeded. The cells were then incubated with 100 μ l of serum-free medium containing 5 μ M DCFH-DA solution for 30 min at 37 °C. After incubation and being washed twice with PBS buffer, a fluorescence intensity of 485/530 nm was read using a spectrofluorometer (Schimadzu, USA).

Cell viability

Following treatment with edaravone at various concentrations ranging from 10 to 100 μ M, cells were treated with different doses of radiation (1, 2, 3, and 4 Gy). Cell viability was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT reduction), as previously described (Lu et al. 2007). Briefly, MTT was added at a final concentration of 1 mg/mL into serum-free medium and incubated for 2 h in a CO₂ incubator at 37 °C. The resultant insoluble formazan crystals were collected and dissolved using dimethyl sulfoxide (DMSO). The relative value of absorbance, recorded at 570 nm, was used to reflect cell viability.

Determination of antioxidant enzymes

Following the indicated treatment, lymphocytes were suspended in 130 mM KCl plus 50 mM PBS containing 0.1 ml of 0.1 M dithiothreitol. Cells were centrifuged at 10,000 rpm for 15 min at 4 °C and the supernatant was taken for biochemical evaluation. Superoxide dismutase activity was assessed using a procedure based on the inhibition of the formation of the NADH–PMS–NBT complex, as described previously (Kakkar et al. 1984). Catalase activity was measured by quantifying the amount of hydrogen peroxide after reacting with dichromate in acetic acid (Sinha 1972). The total reduced glutathione content was assayed using the Ellman method (Ellman 1959).

TUNEL assay

Apoptotic cells were stained using terminal deoxynucleotidyl transferase-mediated dUTP biotin nick end labeling (TUNEL) method (Wako Pure Chemical, Japan) according to the

manufacturer's instructions. FITC-labeled TUNEL-positive cells were imaged under a fluorescent microscope.

Western blot analysis

Following the indicated treatment, lymphocytes were lysed using cell lysis buffer (Cell Signaling Technology, USA) according to the manufacturer's instructions. A BCA Protein Assay Kit (Pierce, USA) was used to measure protein concentrations. Proteins were subjected to 10 % sodium dodecyl sulfate polyacrylamide gel electrophoresis and electroblotted onto Immobilon-P (Millipore, Billerica, MA), as described previously (Sheng et al. 2012). After being blocked with 5 % nonfat milk in TBS-Tween for 2 h at room temperature, transferred membranes were incubated sequentially with primary antibodies for 3 h at room temperature and secondary antibodies for 2 h at room temperature. Immunoreactive bands were developed using the ECL technique (Santa Cruz Biotechnology, USA) according to the manufacturer's instructions.

Determination of DNA strand breaks by alkaline comet assay

Comet assay was performed under alkaline conditions according to the method previously described (Singh et al. 1988). The measurements of different comet end points, such as % tail DNA, tail length, tail moment, and olive tail moment, were considered and analyzed as previously reported (Lovell and Omori 2008).

Statistical analysis

Data are presented as means $\pm$ standard error of mean (SEM). Statistical analysis of data was performed using SPSS 8.0 software. One way analysis of variance (ANOVA) and Dunnett's significant post hoc test were applied to analyze for differences. A difference of $p < 0.05$ was considered to be a statistically significant difference.

Results

Firstly, we selected the optimum dose of edaravone for radioprotection. We observed 98.2 % viability of lymphocytes using up to 100 μ M of edaravone treatment upon 24 h incubation. When edaravone concentration increased beyond 100 μ M, there was a significant decrease in the viability of human peripheral blood lymphocytes (Fig. 1a). Hence, 100 μ M of edaravone was chosen as optimum dose for radioprotection experiments. Human peripheral blood lymphocytes showed 55 and 68 % cytotoxicity at 3 and 4 Gy of irradiation, respectively (data not shown). The

Fig. 1 Edaravone prevents cell death of human peripheral blood lymphocytes induced by γ -irradiation. **a** The effects of edaravone on cell viability. Human peripheral blood lymphocytes were incubated with edaravone for 24 h, and cell viability was determined by MTT assay (* $p < 0.05$ vs without edaravone group; ** $p < 0.01$ vs without edaravone group, $n = 4$). **b** Reduction in percentage of cell survival at 24 h post-irradiation (1, 2, 3, 4, and 5 Gy), with and without edaravone (100 μ M) in human blood lymphocytes (* $p < 0.05$ vs. within the group without edaravone treatment; ** $p < 0.01$ vs. within the group without edaravone treatment, $n = 4$). **c** Edaravone enhances cell survival at concentrations ranging from 10 to 100 μ M in a dose-dependent manner at 24 h post-irradiation (3 Gy irradiation) (* $p < 0.05$ vs. without edaravone group; ** $p < 0.01$ vs. without edaravone group, $n = 4$)

LD50 values were calculated and 3 Gy was used as the optimum dose for further experimentation. MTT assay results showed that exposing lymphocytes to graded radiation doses 1, 2, 3, 4, and 5 Gy caused 82, 63, 46, 35, and 21 % cell survival, respectively (Fig. 1b). However, pretreatment with 100 μ M edaravone before 1, 2, 3, 4, and 5 Gy irradiation showed 91, 81, 68, 58, and 45 % cell survival, respectively. Meanwhile, pretreatment with edaravone at

Fig. 2 Eदारavone prevents oxidative stress in human peripheral blood lymphocytes induced by γ -irradiation. **a** ROS was measured by DCFH-DA. Representative fluorescence photos of intracellular ROS and quantitative analysis of increased intracellular ROS accumulation in γ -irradiated human peripheral blood lymphocytes, which was inhibited by administration of edaravone (* p <0.01 vs. control group; # p <0.01 vs. γ -irradiation group, error bars come from different images from one

experiment, experiments were repeated four times); **b** levels of SOD activity (* p <0.01 vs. control group; # p <0.01 vs. γ -irradiation group, n =4); **c** levels of glutathione peroxidase activity (* p <0.01 vs. control group; # p <0.01 vs. γ -irradiation group, n =5); **d** the levels of reduced glutathione (* p <0.01 vs. control group; # p <0.01 vs. γ -irradiation group, n =4)

concentrations ranging from 10 to 100 μ M protected cells from 3 Gy irradiation in a dose-dependent manner (Fig. 1c).

Levels of ROS in lymphocytes were determined by DCFH-DA assay. After exposure to 3 Gy of irradiation, a significant increase in ROS levels was observed in lymphocytes. However, pretreatment with edaravone significantly inhibited intracellular ROS production in 3 Gy irradiated lymphocytes (Fig. 2a). The activities of superoxide dismutase and glutathione peroxidase play critical roles in maintaining the redox balance in cells. Our results indicate that the activities of these antioxidant enzymes decrease significantly in γ -irradiated lymphocytes. Pretreatment with edaravone significantly increased superoxide dismutase (Fig. 2b) and glutathione peroxidase (Fig. 2c) activities in irradiated lymphocytes. Levels of nonenzymatic antioxidant as reduced glutathione were found to be decreased in γ -irradiated lymphocytes. Pretreatment with 100 μ M significantly restored the reduced glutathione levels in the γ -irradiated lymphocytes (Fig. 2d).

The patterns of apoptosis in γ -irradiated lymphocytes were accessed using the TUNEL method. As shown in Fig. 3a, b, there were few TUNEL-positive cells in normal lymphocytes. By contrast, the ratio of TUNEL-positive cells was notably increased in γ -irradiated lymphocytes (p <0.01). However, pretreatment with edaravone could evidently decrease the number of TUNEL-positive cells in γ -irradiated lymphocytes (p <0.01). Previous studies have demonstrated that exposure to γ -irradiation induced changes in Bax and Bcl-2 expression. Our results indicate an inhibitory effect on γ -irradiation-induced changes in Bax and Bcl-2 gene expression (Fig. 4a, b). Our results also demonstrate that caspase-3 is activated in γ -irradiated lymphocytes. However, activation of caspase-3 was significantly inhibited by edaravone treatment (Fig. 5a). Consistently, the level of cleaved PARP in γ -irradiated lymphocytes was elevated, but this effect was ameliorated by treatment with edaravone (Fig. 5b).

DNA damage is induced by free radicals generated by γ -irradiation. In order to assess whether pretreatment with edaravone has a protective effect against γ -irradiation-

Fig. 3 Apoptosis in human peripheral blood lymphocytes following edaravone pretreatment and γ -irradiation exposure. **a** After edaravone pretreatment and γ -irradiation exposure, apoptotic cells were stained using a TUNEL Assay Kit. Nuclear DNA was stained with DAPI; **b**

quantitative analysis of apoptotic human peripheral blood lymphocytes (* p <0.01 vs. control group; # p <0.01 vs. γ -irradiation exposure group, one-way ANOVA, error bars come from different images from one experiment, experiments were repeated four times)

Fig. 4 Expression of pro-apoptotic protein Bax and anti-apoptotic Bcl2 in human peripheral blood lymphocytes following edaravone pretreatment and γ -irradiation exposure. **a** Representative images of immunoblots for Bax and Bcl-2. Expression of β -actin was used as a loading

control; **b** quantitative analysis of the ratio of Bax and Bcl-2 (* p <0.01 vs. control group; # p <0.01 vs. γ -irradiation exposure group, one-way ANOVA, n =4)

induced DNA damage, changes in the levels of comet parameters (% tail DNA, tail length, tail moment, and olive tail moment) were investigated. The results are shown in Table 1. γ -irradiated lymphocytes showed increased levels of DNA damage indices. Treatment with 100 μ M edaravone 2 h prior to 3 Gy γ -irradiation was able to protect cellular DNA in lymphocytes.

Discussion

It is well known that radiation is one of the most severe causes of oxidative stress in living cells mediated by the flux of free radicals (Riley 1994). Ionizing radiation can generate ROS, such as superoxide anion ($O_2^{\bullet-}$), hydroxyl radical ($OH^{\bullet}$), and hydrogen peroxide (H_2O_2), by splitting water molecules. It has been well established that this production of ROS is the critical determinant of toxicity associated with exposure to ionizing radiation. The most important cellular targets for radiation-induced free radicals are DNA, proteins, and lipids (Shirazi et al. 2007; Robbins and Zhao 2004). Since the use of radiation in diagnosis and treatment has been extended so rapidly in recent years, the discovery of effective radioprotectors that can protect living systems from onslaughts of ionizing radiation is of paramount importance in radiation biology. These radioprotectors should have

characteristics such as the ability to mitigate injuries caused by radiation-induced highly reactive free radicals and they should not produce cumulative or irreversible toxicity.

In the present study, we demonstrated that edaravone effectively protects human blood lymphocytes against potentially lethal levels of irradiation and prolongs the survival time of lethally irradiated cells. In an acute toxicity study, treatment using up to 100 μ M edaravone did not induce any cytotoxicity in human blood lymphocytes. In a cell survival study, lymphocytes in the irradiated group exhibited signs of apoptotic cell death within 24 h after exposure to 3 Gy γ -irradiation. Importantly, pretreatment with edaravone (100 μ M) mitigated the production of ROS induced by 3 Gy γ -irradiation. Additionally, edaravone is known to block both the formation of free radicals and the conversion of endogenous antioxidant enzymes (Wang et al. 2011). Consistently, pretreatment with edaravone was able to rescue the impaired endogenous antioxidant enzymes superoxide dismutase and catalase, which are responsible for the detoxification of deleterious oxygen radicals. Subsequently, edaravone maintained the levels of reduced glutathione in γ -irradiated human blood lymphocytes, which is consistent with the previous study (Li et al. 2013).

Different types of genetic damage have been found to be caused by ionizing radiation. These types of damage include base modifications to single- and double-stranded DNA

Fig. 5 Effects of edaravone on levels of cleaved caspase-3 and PARP as measured by Western blot analysis. **a** Edaravone treatment attenuates γ -irradiation-induced activation of caspase-3; **b** edaravone treatment

attenuates γ -irradiation-induced activation of PARP (* p <0.01 vs. control group; # p <0.01 vs. γ -irradiation exposure group, n =4)

Table 1 Effect of edaravone on radiation-induced DNA damage as measured by comet assay (% tail DNA, tail length, tail moment, olive tail moment) in human peripheral blood lymphocytes. Values are expressed as means $\pm$ SD of six experiments in each group

Group	% tail DNA	Tail length	Tail movement	Olive tail movement
Sham group	1.31 $\pm$ 0.1	6.1 $\pm$ 0.4	0.16 $\pm$ 0.02	0.23 $\pm$ 0.03
Radiation only	23.2 $\pm$ 0.9***	76 $\pm$ 7.2***	16.25 $\pm$ 0.6***	19.28 $\pm$ 0.6***
Edaravone+ γ -radiation	10.3 $\pm$ 0.8 ###	41 $\pm$ 3.6###	7.6 $\pm$ 0.5###	8.3 $\pm$ 0.5###

*** p <0.001 compared to the sham group; ### p <0.001 compared to the radiation-only group

breaks (Natarajan et al. 1980). In the current study, we observed significant DNA damage parameters as measured by comet assay (% tail DNA, tail length, tail moment, and olive tail moment) in 3 Gy of irradiated human peripheral blood lymphocytes (Table 1). Conversely, treatment of cells with edaravone before radiation exposure significantly decreased comet attributes, which may imply that edaravone possesses DNA repair capacity in irradiated human peripheral blood lymphocytes. Since DNA damage induced by radiation is the result of the generation of ROS, a compound with antioxidant potential can scavenge ROS and ameliorate DNA damage. Edaravone is known to enhance DNA repair mechanisms and inhibit DNA strand breaks against ROS (Takizawa et al. 2009). Many synthetic compounds have been investigated in the recent past; nonetheless, the ones that are currently available present many drawbacks including high cost, undesirable side effects, and toxicity (Maurya et al. 2006). As a licensed clinical drug, the safety of edaravone has been guaranteed. In this study, we found that edaravone protects human peripheral blood lymphocytes against radiation damage from multiple perspectives. Hence, we extended our aim to study its effects on protecting cells from radiation in cultured human peripheral blood lymphocytes. Edaravone is a potent and novel synthetic scavenger of free radicals, which inhibits both nonenzymatic lipid peroxidation and the lipoxygenase pathway. Notably, edaravone readily crosses the blood–brain barrier (BBB), which is a major advantage in clinical use (Lapchak 2010). In addition to neutralizing multiple ROS and protecting both neurons and endothelial cells in the neurovascular unit, edaravone also exerts anti-apoptotic and anti-inflammatory actions that contribute to cytoprotection in ischemic stroke (Zhang et al. 2005). Moreover, edaravone reduced mitochondrial damage in homogenate made from ischemic-incubated rat brain (Watanabe et al. 1994). As compared with other antioxidants, edaravone has the following advantages: (1) it has already been used in clinical practice mostly without the presence of serious side effects at the dose currently applied (Sueishi et al. 2002); (2) it is lipophilic, and therefore, readily accessible to tissues (Sato et al. 2003); (3) it preferentially

accumulates in the kidneys (Spitz et al. 2011). This accumulation in the kidneys is desirable because it presumably confers some selectivity towards the healthy tissue and favors nephroprotection.

The main limitation of this study is that its findings are based on in vitro experiments. In vivo studies are a vital part of the development of new treatment methods using medications. Animal or human studies will help us to better understand how treatment with edaravone interacts throughout the whole bodies of patients exposed to γ -radiation, rather than isolated areas. Testing the effects of edaravone on peripheral blood lymphocytes in vivo and visualizing edaravone efficacy in vivo are our future targets of research. Another limitation of this study is that the mechanisms underlying the protective effects of edaravone against γ -radiation are still unknown. In the future, we will focus on a more detailed mechanistic study of the factors that influence the results of edaravone treatment.

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