Free Radical Scavenger Edaravone Suppresses X-ray-induced Apoptosis through p53 Inhibition in MOLT-4 Cells

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Edaravone, a clinical drug used widely for the treatment of acute cerebral infarction, is reported to scavenge free radicals. In the present study, we investigated the radioprotective effect of edaravone on X-ray-induced apoptosis in MOLT-4 cells. Apoptosis was determined by the dye exclusion test, Annexin V binding assay, cleavage of caspase, and DNA fragmentation. We found that edaravone significantly suppressed the X-ray-induced apoptosis. The amount of intracellular ROS production was determined by the chloromethyl-2',7'-dichlorodihydro-fluorescein diacetate system. We found that the intracellular ROS production by X-irradiation was completely suppressed by the addition of edaravone. The accumulation and phosphorylation of p53 and the expression of p21WAF1, a target protein of p53, which were induced by X-irradiation, were also suppressed by adding edaravone. We conclude that the free radical scavenger edaravone suppresses X-ray-induced apoptosis in MOLT-4 cells by inhibiting p53.

INTRODUCTION

X-ray-induced cell death results from two types of actions, direct and indirect.1,3 In the first, the X-rays directly ionize or excite macromolecules in the cells, leading to cell damage. In the second, the X-rays excite water molecules in the cells and produce reactive oxygen species (ROS), which damage the cells. Approximately 70% of the biological damage caused by X-rays results from this indirect action.2 Cells that are critically damaged by X-rays will die either by interphase cell death or reproductive cell death.3 Most of the X-ray-induced cell deaths observed in thymocytes are of the interphase type, known as apoptosis.4,5 ROS are postulated to play a central role in X-ray-induced apoptosis,6 with the hydroxyl radical being the most important. Thus, agents that could suppress ROS would be expected to protect cells from X-ray-induced apoptosis and improve cell survival.

Edaravone (MCI-186; 3-methyl-1-phenyl-2-pyrazolin-5-one; Radicut) is a clinical drug that is used widely for the treatment of acute cerebral infarction. Its effectiveness as a treatment for this condition has been reported in many studies, including in vivo,7–20 in vitro,21,22 and clinical settings.23–26 Edaravone scavenges free radicals as an electron donor27–29; therefore, it seemed likely to be useful for radioprotection.

Indeed, in a previous study, Anzai and colleagues reported that the intraperitoneal administration of edaravone to mice increased the lethal dose of radiation for the animals,30 indicating that the drug has a radioprotective effect. In that report, edaravone’s radioprotective effect was probably due mainly to the suppression of bone-marrow syndrome, because the X-ray dose used in the experiment was under 10 Gy.30 However, edaravone’s radioprotective mechanism is not fully understood at the molecular level.

Bone-marrow syndrome occurs mainly as a result of the apoptosis of stem cells.31 In the present study, we investigated the effect of edaravone on the apoptosis of MOLT-4 cells after X-irradiation. The human T-cell leukemia cell line MOLT-4 is highly sensitive to X-rays; after X-irradiation it undergoes an apoptotic cell death that is characterized by nuclear condensation and DNA fragmentation and is mediated by activated caspases.32–35 Recent studies have demonstrated that the p53 and JNK pathways are involved in the radiation-induced apoptosis of MOLT-4 cells.34–37 The results presented here suggest that edaravone suppresses the X-ray-induced apoptosis in MOLT-4 cells by inhibiting p53 and caspase.

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MATERIALS AND METHODS

Cell culture
Human T-cell leukemia MOLT-4 cells were cultured in suspension with RPMI-1640 medium (Sigma) containing 5% fetal bovine serum (Hyclone) and antibiotics (100 units/ml of penicillin/streptomycin), and incubated at 37°C in a humidified atmosphere of 5% CO₂ and 95% air.

Chemicals
Edaravone was kindly provided by the Mitsubishi Pharma Corporation (Tokyo, Japan). We dissolved 52.5 mg edaravone in 192.5 μl of 2 M NaOH and 1.05 ml of DDW, and then adjusted the pH to 8.8 with 2 M HCl. Finally, physiological saline was added to adjust the final concentration of edaravone to 30 mg/ml.

X-irradiation
X-irradiation was performed with an X-ray generator (Pantak HF 350, Shimadzu) at 200 kVp and 20 mA, with a filter of 0.5 mm Cu and 1 mm Al, and at a dose rate of 1.35–1.40 Gy/min.

Dye exclusion test
One hundred microliters of cell suspension (approximately 5 × 10⁵ cells/ml) was mixed with 25 μl of 1% erythrosin B in phosphate-buffered saline (PBS). The numbers of stained (dead) cells and unstained (live) cells were counted and the viability (%) was calculated as follows:

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\text{Viability (\%)} = \left(\frac{\text{number of unstained cells}}{\text{total cell number}}\right) \times 100
\]

Annexin V Binding Assay
The extent of apoptosis was determined by Annexin V-FITC and propidium iodine (PI) staining, using the MEB-CYTO Apoptosis Kit (MBL). Flow cytometric analysis was carried out with an EPICS flow cytometer (XL System II, Beckman Coulter), using a single laser emitting excitation light at 488 nm. In the FITC/PI diparametric plot, quadrants 1 (lower FITC/ upper PI), 2 (upper FITC/ upper PI), 3 (lower FITC/ lower PI), and 4 (upper FITC/ lower PI) represent the fractions of secondary-necrotic, primary-necrotic, viable, and apoptotic cells, respectively. More than 5,000 cells were subjected to the analysis.

Quantification of intracellular ROS
The amount of intracellular ROS production was measured by chloromethyl-2′, 7′-dichlorodihydro-fluorescein diacetate (CM-H₂-DCFDA, Molecular Probes). MOLT-4 cells were incubated in the dark with approximately 5 μg/ml of probe CM-H₂-DCFDA for an hour, and the fluorescence intensity was analyzed by an EPICS flow cytometer (XL System II, Beckman Coulter) using a laser excitation and emission wavelength of 492–495 nm and 517–527 nm, respectively.

Western blot analysis
Cells were lysed in a sodium dodecyl sulfate (SDS) sample buffer (1% SDS, 3% β-mercaptoethanol, 5% glycerol, 62.5 mM, Tris-HCl, pH 6.8). Proteins were separated by 10% or 15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred onto polyvinylidene difluoride membranes (Immobilon, Millipore). After blocking for 30
minutes in 5% skim milk in Tris-buffered saline (TBS, 29 mM Tris-HCl, 0.9% NaCl, pH 7.6) supplemented with 0.05% Tween-20 (TBS-T), the membranes were incubated overnight at 4°C in TBS-T containing 5% skim milk and primary antibodies. The primary antibodies were anti-p53 (clone DO-1, Santa Cruz Biotechnology), anti-phospho p53 at Ser 15 (Calbiochem), anti-cleaved caspase-3 (Cell Signaling), anti-caspase-7 (MBL), anti-p21WAF1 (Calbiochem), and anti-Bcl-2 (Pharmingen). After being rinsed with TBS-T three times, the membranes were incubated overnight at 4°C in TBS-T containing 5% skim milk and secondary antibodies conjugated with horseradish peroxidase (DAKO). The membranes were then washed three times with TBS-T, once with TBS (20 mM Tris-HCl, pH 7.5, 150 mM NaCl), and developed using an ECL-plus kit (Amersham Biosciences). The signals were obtained by exposure to X-ray.

![Diagram](Image)

**Fig. 2.** Effect of edaravone on apoptosis, determined by Annexin V-PI staining. (A) MOLT-4 cells were subjected to 5 Gy X-ray irradiation without edaravone treatment. (B) MOLT-4 cells were subjected to 5 Gy X-ray irradiation 5 minutes after the addition of 3 mg/ml edaravone. Typical flow cytometry results of Annexin V-PI staining performed 16 hours after X-ray irradiation are shown. The transverse axis shows Annexin V-stained cells and the vertical axis shows PI-stained cells. (C) The percentage of cells stained by Annexin V and unstained by PI which is interpreted as in the early stage of apoptosis, is shown. MOLT-4 cells were harvested 16 hours after treatment (5 Gy X-ray irradiation and/or 3 mg/ml edaravone 5 minutes before X-ray irradiation). (D) The percentage of cells unstained both by Annexin V and PI is shown. MOLT-4 cells were harvested 20 hours after treatment (5 Gy X-ray irradiation and/or 3 mg/ml edaravone 5 minutes before X-ray irradiation). *p < 0.05.
films (Hyperfilm MP, Amersham Biosciences).

**Analysis of DNA fragmentation**

Approximately 1 × 10⁶ of control or treated cells were harvested at the indicated time points. DNA was extracted using the Apoptosis Ladder Detection Kit (WAKO), according to the manufacturer’s instructions. The DNA pellet was washed, resuspended, and subjected to electrophoresis on a 1.5% agarose gel at 100 volts for 30 minutes. The gel was visualized by staining with 1 μg/ml ethidium bromide and observed under a UV transilluminator and photographed.

**Statistical analysis**

All experiments were repeated at least three times. The results are expressed as the mean ± standard deviation (SD) of the mean. All laboratory data were evaluated according to standard statistical methods, using commercially available computer programs such as Microsoft Excel 2000. Statistical differences were determined using the Student’s t-test. In all tests, p values less than 0.05 were considered statistically significant.

**RESULTS**

**Effects of edaravone on X-ray-induced cell death**

First, to determine the optimal concentration of edaravone to use in the experiments, we investigated its cytotoxicity using the dye exclusion test. The cell viability was examined in cultures treated with 0.15, 0.75, 1.5, 3, and 6 mg/ml edaravone (Fig. 1A). At concentrations of edaravone less than 3 mg/ml, the cell viability was more than approximately 60%, which was considered acceptable. A dose of 6 mg/ml, however, proved cytotoxic for MOLT-4 cells (Fig. 1A). Thus, we performed the following experiments using a concentration of 3 mg/ml.

To examine the effects of edaravone on X-ray-induced cell death, we determined the time course of cell viability after 5 Gy X-irradiation with or without 3 mg/ml edaravone, using the dye exclusion test. When MOLT-4 cells were irradiated without edaravone, the cell viability 4, 8, 12, 16, and 20 hours after X-irradiation was 93.3 ± 1.7%, 67.8 ± 3.4%, 16.5 ± 1.2%, 9.6 ± 1.4%, and 7.7 ± 0.8%, respectively (Fig. 1B). When edaravone was added 5 minutes before the X-irradiation, the cell viability was 92.8 ± 1.4%, 92.6 ± 2.4%, 89.2 ± 2.0%, 75.8 ± 2.6%, and 45.6 ± 4.1%, respectively (Fig. 1B). The cell viability with edaravone was significantly higher from 8 to 20 hours after X-irradiation than that of cells that were not treated with edaravone (p < 0.05). These data indicate that edaravone significantly inhibited the X-ray-induced cell death of MOLT-4 cells. We also performed the same examination with 1.5 mg/ml edaravone, however, the cell viability did not increase significantly when 1.5 mg/ml edaravone was added 5 minutes before X-irradiation (data not shown). We considered that less than 1.5 mg/ml edaravone had no effect on the MOLT-4 cell viability after X-irradiation.

Next, we examined the effect of the radiation dose on the cell viability after X-irradiation. MOLT-4 cells were untreated or treated with 3 mg/ml edaravone, then subjected to 2 or 5 Gy X-irradiation 5 minutes later. The dye exclusion test was performed 20 hours after X-irradiation. The cell viability after 2 and 5 Gy X-irradiation without edaravone was 36.7 ± 1.7% and 7.7 ± 0.8%, respectively (Fig. 1C). The cell viability after 2 and 5 Gy X-irradiation with edaravone treatment was 45.5 ± 2.9% and 45.6 ± 4.1%, respectively (Fig. 1C). The cell viability at X-ray doses of 2 and 5 Gy was significantly improved by the addition of edaravone (p < 0.05).

Next, we examined the effect of the edaravone added after X-irradiation on the cell viability. MOLT-4 cells were subjected to 5 Gy X-irradiation, then untreated or treated with 3 mg/ml edaravone 4 hours later. The dye exclusion test was performed 20 hours after X-irradiation. The cell viability was partially improved when edaravone was added 4 hours after X-irradiation (data not shown).

**Effects of edaravone on apoptosis**

To assess the effect of edaravone on X-ray-induced apoptosis, we performed Annexin V-PI staining 16 or 20 hours after X-irradiation by flow cytometry. The appearance of Annexin V+/PI– cells, which were interpreted as in the early stage of apoptosis, was significantly suppressed by the addition of 3 mg/ml edaravone 5 minutes before X-irradiation (p < 0.05) (Fig. 2A–C). The percentage of Annexin V–/PI– cells, which were interpreted as viable, was 10.6 ± 0.8% when the cells were irradiated without 3 mg/ml edaravone. When MOLT-4 cells were irradiated with or without 3 mg/ml edaravone, then subjected to 2 or 5 Gy X-irradiation 5 minutes later. The dye exclusion test was performed 20 hours after X-irradiation. The cell viability was more than approximately 60%.

**Fig. 3.** Intracellular ROS determined by the CM-H₂DCFDA flow cytometry system. The amount of intracellular ROS after treatment (20 Gy X-irradiation with or without 3 mg/ml edaravone) is shown. Edaravone was added 5 minutes before or after X-irradiation. The ROS production of each sample was quantified as described in Materials and Methods. Data shown are means ± SD from at least three independent experiments. *p < 0.05.
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and 74.2 ± 2.1% when they were X-irradiated with edaravone (p < 0.05) (Fig. 2D). These data indicate that the radioprotective effect of edaravone is due to the suppression of apoptosis.

We also examined the effect of 3 mg/ml edaravone added 4 hours after X-irradiation on X-ray-induced apoptosis. Annexin V-PI staining was performed 16 hours after 5 Gy-X-irradiation. The appearance of Annexin V+/PI– cells was partially suppressed by the addition of 3 mg/ml edaravone 4 hours after X-irradiation (data not shown).

Effects of edaravone on the production of intracellular ROS

To examine the effect of edaravone on the X-ray-induced production of intracellular ROS, we used the CM-H$_2$DCFDA flow cytometry system.$^{38}$ CM-H$_2$DCFDA is a fluorescence-based probe that was recently developed to detect the intracellular production of ROS. CM-H$_2$DCFDA

![Time course of the effects of edaravone (E) on apoptosis-related proteins. MOLT-4 cells were untreated or treated with 3 mg/ml edaravone, then subjected to X-irradiation at 5 Gy 5 minutes later. Proteins were detected by immunoblotting. (A) Effects of edaravone on the accumulation and phosphorylation on Ser 15 of p53 and the induction of p21$^{WAF1}$, a p53 target gene, after X-irradiation. (B) Effects of edaravone on apoptosis-related proteins Bcl-2 and caspase-7.](image)

Fig. 4. Time course of the effects of edaravone (E) on apoptosis-related proteins. MOLT-4 cells were untreated or treated with 3 mg/ml edaravone, then subjected to X-irradiation at 5 Gy 5 minutes later. Proteins were detected by immunoblotting. (A) Effects of edaravone on the accumulation and phosphorylation on Ser 15 of p53 and the induction of p21$^{WAF1}$, a p53 target gene, after X-irradiation. (B) Effects of edaravone on apoptosis-related proteins Bcl-2 and caspase-7.
diffuses passively into cells, is trapped inside, and is deacetylated by intracellular esterases. It is subsequently oxidized to a fluorescent product in the presence of intracellular ROS. The oxidation of CM-H$_2$-DCFDA can be monitored as a convenient determinant of the level of intracellular oxidative stress. X-irradiation at 20 Gy induced an approximately 11-fold increase in basal CM-H$_2$-DCFDA fluorescence (p < 0.05), which was completely suppressed by adding 3 mg/ml edaravone 5 minutes before X-irradiation (Fig. 3). When 3 mg/ml edaravone was added 5 minutes after X-irradiation, however, the basal CM-H$_2$-DCFDA fluorescence did not decrease significantly (Fig. 3). These data suggest that edaravone eliminates the short-term intracellular ROS generated by X-irradiation.

Effects of edaravone on apoptosis-related proteins

We next investigated the effect of edaravone on the accumulation of p53 and on the phosphorylation of p53 at Ser 15 after X-irradiation, by immunoblotting. Fig. 4A shows that both the accumulation of p53 and its phosphorylation at Ser 15 were apparent 1 hour after X-irradiation, and both were suppressed by 3 mg/ml edaravone. Next, we investigated the expression of the p53 target gene, p21$^{WAF1}$. The expression of p21$^{WAF1}$ was apparent 8 hours after X-irradiation, and this expression was inhibited by 3 mg/ml edaravone (Fig. 4A).

We further investigated the effect of edaravone on caspase-3, caspase-7, and Bcl-2 after X-irradiation. The cleavage of caspase-3 was detectable 8 hours after X-irradiation, and this induction was almost completely suppressed by adding 3 mg/ml edaravone (data not shown). The cleavage of caspase-7 induced by X-irradiation was also suppressed by the addition of 3 mg/ml edaravone (Fig. 4B). On the other hand, Bcl-2, which is a known anti-apoptotic protein, was not overexpressed in response to edaravone addition, suggesting that Bcl-2 might not be responsible for the inhibition of apoptosis by edaravone. The cleavage of Bcl-2 was induced 16 hours after X-irradiation, and this cleavage was suppressed by edaravone addition (Fig. 4B), consistent with Bcl-2’s status as a substrate molecule for caspase-3. These data indicate that the addition of edaravone before X-irradiation affects the p53 pathway and caspase activation, but not Bcl-2 overexpression.

Effects of edaravone on DNA fragmentation

DNA fragmentation is a hallmark of apoptosis. It is induced by the activation of caspases, including caspase-3. We examined the effect of edaravone on DNA fragmentation in MOLT-4 cells after X-irradiation. DNA fragmentation was detectable 8 hours after irradiation, and was almost completely suppressed by the addition of 3 mg/ml edaravone (Fig. 5), confirming that the activation of caspase was suppressed by edaravone. The electrophoretic pattern of DNA extracted from MOLT-4 cells irradiated with 5 Gy without addition of edaravone showed a smear pattern, not a ladder pattern, which is compatible with the observation of Akagi and colleagues.

**DISCUSSION**

We found that edaravone suppressed X-ray-induced cell death in vitro (Fig. 1). This finding is consistent with the results of a previous in vivo study, in which the lethal dose of X-irradiation for mice increased after the administration of edaravone. In addition, we found that this radioprotective effect is due to the suppression of apoptosis (Fig. 2). Previous reports indicated that ROS play a crucial role in the induction of apoptosis. We therefore investigated the amount of ROS after X-irradiation with or without edaravone addition, and found that the ROS were significantly suppressed when edaravone was added 5 minutes before X-irradiation, whereas the suppression was not significant when the drug was added 5 minutes after X-irradiation (Fig. 3). This result supports the previous finding that edaravone added after X-irradiation is ineffective as a radioprotector in vivo. Edaravone, which exists as an anion in solution, provides an electron to ROS generated by X-irradiation and inactivates them. One of the most important ROS is the hydroxyl radical, which reacts with biological components immediately upon being generated, and diminishes soon thereafter. Adding edaravone 5 minutes after X-irradiation might be too late to scavenge hydroxyl radicals generated by the X-irradiation, which could explain its lack of effectiveness at this time point. We propose that edaravone suppresses X-ray-induced apoptosis mainly by scavenging ROS. However, X-ray-induced apoptosis was partially suppressed even when edaravone was added 4 hours after X-irradiation. Other mechanisms may be related to the suppression of apoptosis, however, and further investigation is needed.
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p53 is a transcription factor that is well-known to be involved with the cell’s decision between apoptosis and other fates after X-irradiation. After DNA damage, p53’s stability is increased by phosphorylation, and the accumulated p53 induces the transcription of its target genes, one of which is a kinase inhibitor, p21WAF1.34 The over-expression of a dominant-negative form of p53 in MOLT-4 cells results in a resistance of the cells to radiation-induced apoptosis.33 We found that edaravone suppressed the X-ray-induced accumulation of p53 and its phosphorylation at Ser 15 (Fig. 4A). The expression of p21WAF1 after X-irradiation was also suppressed by edaravone, confirming that it inhibited the X-ray-induced p53 activation (Fig. 4A).

Caspases are a family of aspartate-specific cysteine proteases that are activated during apoptosis. They are normally present in cells as proenzymes and require limited proteolysis for activation of their enzymatic activity. Activated caspases precipitate the irreversible commitment of the cell to apoptotic death by cleaving a number of substrates, one of which is Bcl-2.45–47 Bcl-2 is an integral membrane protein that inhibits the apoptosis induced by various stimuli, including heat shock, serum depletion, and chemotherapy agents.40 We previously reported that MOLT-4 cells transfected with mouse Bcl-2 (MOLT-4/mbcl-2) are resistant to X-rays; that is, X-ray-induced apoptosis/rapid cell death was significantly suppressed in the Bcl-2-transfected cells.34 It is reported that the loop domain of Bcl-2 is cleaved at Asp 34 by caspase-3 in vitro, and the carboxyl-terminal Bcl-2 cleavage product is pro-apoptotic.49 In this study, the cleavage of caspase-3 and caspase-7 induced by X-irradiation was suppressed by the prior addition of edaravone (data not shown, Fig. 4B). The findings that edaravone suppresses the activation of p53 and the cleavage of caspase-3 and caspase-7 could be explained by its suppression of ROS. In contrast, the expression of Bcl-2, an anti-apoptotic protein, did not change with the addition of edaravone before X-irradiation (Fig. 4B). This observation is inconsistent with some previous reports, in which the expression of Bcl-2 was increased by edaravone in cerebral ischemic models in vivo,50 and in vitro.51 The discrepancy between the present results and those of previous studies in vivo might be related to differences between the in vitro and in vivo conditions. The discrepancy may also be due to differences in the genetic background of the cells used, MOLT-4 vs. PC12, and/or in the apoptotic stimuli used, X-rays vs. oxygen-glucose deprivation.51 Another previous report suggested that the X-ray-induced apoptosis in MOLT-4 cells is fully p53-dependent.52

Several compounds have been shown to protect living cells from the deleterious effects of X-irradiation. The reported mechanisms of radioprotection, however, differ from compound to compound. For instance, vanadate directly suppresses p53 transactivation, although its effect on ROS has not been investigated. Various antioxidants, including alpha lipoic acid or carboxycysteine-lysine salt, amifostine, reduced glutathione, and vitamin A plus vitamin E plus Vitamin C, all suppressed ROS in vivo.52 Inanami and colleagues reported that a vitamin E analogue, Trolox, which is reported to inhibit lipid peroxidation,53 suppresses the X-ray-induced apoptosis of MOLT-4 cells by inhibiting the caspase-3-dependent pathway.54 Edaravone is also reported to inhibit lipid peroxidation,11,19,55,56 and we found here that it suppressed p53 and caspase activation. Amifostine is a clinical drug with cytoprotective activity against the adverse effects of radiotherapy and chemotherapy in normal tissues; this cytoprotection is attributed to its radioprotective ability to scavenge free radicals57 and to its antimutagenic effects.58 These similar and dissimilar mechanisms of the suppression of apoptosis by various agents are still controversial.

Taking our findings together, we conclude that edaravone scavenges ROS generated by X-irradiation, which suppresses the activation of the p53- and caspase-mediated apoptotic pathway and of DNA fragmentation, and, thus, suppresses X-ray-irradiation-induced apoptosis. Since malignant tumors often are hypoxic, edaravone might protect only normal tissues, not malignant tumors, from X-ray-induced cell damage in radiation therapy.

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