Protective Effect of Triphlorethol-A from *Ecklonia cava* against Ionizing Radiation *in vitro*

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Radiation protection/Oxidative stress/Reactive oxygen species.

We studied the cytoprotective effect of triphlorethol-A against γ-ray radiation-induced oxidative stress. In this study, hydrogen peroxide, which is a reactive oxygen species (ROS), was detected using 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA) assay. Triphlorethol-A reduced intracellular hydrogen peroxide generated by γ-ray radiation. This compound provided protection against radiation-induced membrane lipid peroxidation and cellular DNA damage which are the main targets of radiation-induced damage. Triphlorethol-A protected the cell viability damaged by the radiation through inhibition of apoptosis. Triphlorethol-A reduced the expression of bax and activated caspase 3 induced by radiation, but recovered the expression of bcl-2 decreased by radiation. Taken together, the results suggest that triphlorethol-A protects cells against oxidative damage induced by radiation through reducing ROS.

**INTRODUCTION**

Radiation induces cell damage through the formation of ROS.¹ Sulphhydryl agents such as cysteine, glutathione, β-mercaptoethylamine (cysteamine), and other antioxidants have been shown to protect mice against the lethal effects of radiation. Increased understanding of the interrelationship between the effects of oxygen and radiation exposure leads to the rational application of naturally occurring antioxidants.² Recently, we reported that triphlorethol-A, derived from *Ecklonia cava*, decreased H₂O₂-induced cell damage through activation of an antioxidant system.³ *Ecklonia cava* is a brown alga (Laminariaceae) that is abundant in the subtidal regions of Jeju Island, Korea. It has been reported that *Ecklonia* species exhibit radical scavenging activity,⁴,⁵ antiplasmin inhibiting activity,⁶-⁸ antimutagenic activity,⁹-¹¹ bactericidal activity,¹² HIV-1 reverse transcriptase and proteinase inhibitory activity.¹³,¹⁴ Phlorotannins (polymers of phloroglucinol) have been identified as being responsible for the biological activities of *Ecklonia*. Triphlorethol-A, an open-chain trimer of phloroglucinol, is a phlorotannin component that is isolated from *E. cava*.

In this study, we investigated the protective effects of triphlorethol-A on cell damage induced by γ-ray radiation and the possible mechanism of the cytoprotection.

**MATERIALS AND METHODS**

**Preparation of Triphlorethol-A**

Dried *Ecklonia cava* (4 kg), collected from Jeju Island, Korea, was immersed in 80% methanol at room temperature for 2 days. The aqueous methanol was removed *in vacuo* to produce a brown extract (1 kg), which was partitioned between ethyl acetate and water. The ethyl acetate fraction (230 g) was mixed with celite. The mixed celite was dried and packed into a glass column, and eluted in following order, with hexane, methylene chloride, diethyl ether, and methanol. The diethyl ether fraction (14 g) was subjected to Sephadex LH-20 chromatography using CHCl₃-MeOH gradient solvent (2/1→0/1). Triphlorethol-A (220 mg) was retrieved from these fractions and was identified according to a previously reported method (Fig. 1).⁸ The purity of triphlorethol-A, which was assessed by HPLC, was > 90%. Triphlorethol-A was then dissolved in dimethylsulfoxide (DMSO); the final concentration of which did not exceed 0.1%.
Reagents

2',7'-dichlorodihydrofluorescein diacetate (DCF-DA), propidium iodide, and Hoechst 33342 were purchased from Sigma Chemical Company, St. Louis, MO, USA. The other chemicals and reagents were of analytical grade.

Cell Culture

Chinese hamster lung fibroblasts (V79-4) cells from the American Type Culture Collection were maintained at 37°C in an incubator with a humidified atmosphere of 5% CO₂ and cultured in Dulbecco’s modified Eagle’s medium containing 10% heat-inactivated fetal calf serum, streptomycin (100 μg/ml) and penicillin (100 units/ml).

Irradiation

Cells were exposed to γ-ray from a ⁶⁰Co γ-ray source (MDS Nordion C-188 standard source, located in Cheju

Fig. 1. Chemical structure of triphlorethol-A.

Fig. 2. Effect of triphlorethol-A on scavenging intracellular ROS generated by γ-ray radiation. The V79-4 cells were treated with triphlorethol-A at 30 μM and 1 h later, γ-ray radiation at 10 Gy was applied to the cells. The intracellular ROS was detected using fluorescence spectrophotometer (A) and flow cytometry (B) after DCF-DA staining. The measurements were made in triplicate and values are expressed as means ± S.E. FI means fluorescence intensity. *Significantly different from 0 Gy (p < 0.05).
National University, Jeju, Korea) at a dose rate of 1.5 Gy/min.

Intracellular Reactive Oxygen Species Measurement
The DCF-DA method was used to detect the intracellular hydrogen peroxide level. DCF-DA diffuses into cells, where it is hydrolyzed by intracellular esterase to polar 2',7'-dichlorodihydrofluorescein. This non-fluorescent fluorescein analog gets trapped inside the cells and is oxidized by intracellular oxidants to a highly fluorescent, 2',7'-dichlorofluorescein. The V79-4 cells were treated with triphlorethol-A at 30 μM and 1 h later, γ-ray radiation at 10 Gy was applied to the cells. The cells were incubated for an additional 24 h at 37°C. After the addition of 25 μM of DCF-DA solution, the fluorescence of 2',7'-dichlorofluorescein was detected using a Perkin Elmer LS-5B spectrofluorometer and using flow cytometry (Becton Dickinson, Mountain View, CA, USA), respectively.

Fig. 3. Protective effect of triphlorethol-A upon γ-ray radiation-induced lipid membrane damage of V79-4 cells. Lipid peroxidation was assayed by measuring the amount of TBARS. The measurement was made in triplicate and values are expressed as means ± S.E. *Significantly different from 0 Gy (p < 0.05).

Fig. 4. Protective effect of triphlorethol-A upon γ-ray radiation-induced cellular DNA damage of V79-4 cells. The cellular DNA damage was detected by an alkaline comet assay. The measurement was made in triplicate and values are expressed as means ± S.E. *Significantly different from 0 Gy (p < 0.05).
Cell Viability

The effect of triphlorethol-A on the viability of the V79-4 cells was determined using the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] bromide (MTT) assay, which is based on the reduction of a tetrazolium salt by mitochondrial dehydrogenase in viable cells.\(^{17}\) The V79-4 cells were treated with triphlorethol-A and \(\gamma\)-ray radiation, and the cells were incubated for 48 h at 37°C. Fifty \(\mu\)l of the MTT stock solution (2 mg/ml) was then added to each well to attain a total reaction volume of 200 \(\mu\)l. After incubating for 4 h, the plate was centrifuged at 800 \(\times\) g for 5 min and the supernatants were aspirated. The formazan crystals in each well were dissolved in 150 \(\mu\)l of DMSO and the \(A_{540}\) was read on a scanning multi-well spectrophotometer.

Lipid Peroxidation Inhibitory Activity

Lipid peroxidation was assayed by a thiobarbituric acid reaction.\(^{18}\) The V79-4 cells were treated with triphlorethol-A at 30 \(\mu\)M and 1 h later, \(\gamma\)-ray radiation at 10 Gy was applied to the cells. The cells were incubated for 24 h at 37°C. The cells were then washed with cold PBS, scraped and homogenized in ice-cold 1.15% KCl. One hundred \(\mu\)l of the cell lysates was mixed with 0.2 ml of 8.1% SDS, 1.5 ml of 20% acetic acid (adjusted to pH 3.5) and 1.5 ml of 0.8% thiobarbituric acid (TBA). Distilled water was added to the mixture to a final volume of 4 ml and heated to 95°C for 2 h. After cooling to room temperature, 5 ml of an \(n\)-butanol and pyridine mixture (15:1, \(v/v\)) was added to each sample, and the mixture was shaken. After centrifugation at 1000 \(\times\) g for 10 min, the supernatant fraction was isolated, and the absorbance was measured spectrophotometrically at 532 nm.

Flow Cytometry Analysis

Flow cytometry was performed in order to determine the apoptotic sub-G\(_1\) hypodiploid cells.\(^{19}\) The V79-4 cells were treated with triphlorethol-A at 30 \(\mu\)M and 1 h later, \(\gamma\)-ray radiation at 10 Gy was applied to the cells. The cells were then incubated for an additional 48 h at 37°C, subsequently harvested, and fixed in 1 ml of 70% ethanol for 30 min at 4°C. The cells were washed twice with PBS, and then incubated for 30 min in the dark at 37°C in 1 ml of PBS containing 100 \(\mu\)g propidium iodide and 100 \(\mu\)g RNase A. Flow cytometric analysis was performed using a FACS Calibur flow cytometer (Becton Dickinson, Mountain View, CA, USA). Sub-G\(_1\) hypodiploid cells were assessed based on the histograms generated using the computer programs, Cell Quest and Mod-Fit.

Nuclear Staining with Hoechst 33342

The V79-4 cells were treated with triphlorethol-A at 30 \(\mu\)M and 1 h later, \(\gamma\)-ray radiation at 10 Gy was applied to the cells. The cells were then incubated for 48 h at 37°C. 1.5 \(\mu\)l of Hoechst 33342 (stock 10 mg/ml), a DNA-specific fluorescent dye, was added to each well (1.5 ml) and incubated for 10 min at 37°C. The stained cells were observed under a fluorescent microscope, which was equipped with a CoolSNAP-Pro color digital camera in order to examine the degree of nuclear condensation.

Single Cell Gel Electrophoresis (Comet Assay)

Comet assay was performed to determine the oxidative DNA damage.\(^{20,21}\) The cell suspension was mixed with 75 \(\mu\)l of 0.5% low melting agarose (LMA) at 39°C and spread on a fully frosted microscopic agarose slide that was pre-coated with 200 \(\mu\)l of 1% normal melting agarose (NMA). After solidification of the agarose, the slide was covered with another 75 \(\mu\)l of 0.5% LMA and then immersed in a lysis solution...
(2.5 M NaCl, 100 mM Na-EDTA, 10 mM Tris, 1% Trion X-100, and 10% DMSO, pH 10) for 1 h at 4°C. The slides were then placed in a gel-electrophoresis apparatus containing 300 mM NaOH and 10 mM Na-EDTA (pH 13) for 40 min to allow DNA unwinding and the expression of the alkali labile damage. An electrical field was then applied (300 mA, 25 V) for 20 min at 4°C to draw negatively charged DNA toward an anode. After electrophoresis, the slides were washed three times for 5 min at 4°C in a neutralizing buffer (0.4 M Tris, pH 7.5) and then stained with 75 µl of propidium iodide (20 µg/ml). The slides were observed with a fluorescence microscope after Hoechst 33342 staining and apoptotic bodies are indicated by arrows. (E) Apoptotic sub-G₁ DNA content was detected by flow cytometry after propidium iodide staining. (F) Western blot analysis was performed using anti-bcl-2, -bax, -caspase 3 and -PARP antibodies.

**Western Blot Analysis**

The V79-4 cells were treated with triphlorethol-A at 30 µM and 1 h later, γ-ray radiation at 10 Gy was applied to the cells.
cells. The cells were then incubated for 48 h at 37°C. The cells were harvested, and washed twice with PBS. The harvested cells were then lysed on ice for 30 min in 100 μl of a lysis buffer [120 mM NaCl, 40 mM Tris (pH 8), 0.1% NP 40] and centrifuged at 13,000 × g for 15 min. Supernatants were collected from the lysates and protein concentrations were determined. Aliquots of the lysates (40 μg of protein) were boiled for 5 min and electrophoresed in 10% SDS-polyacrylamide gel. Blots in the gels were transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA), which were then incubated with anti-primary rabbit monoclonal bcl-2, bax, caspase 3 and PARP antibodies. The membranes were further incubated with goat anti-rabbit immunoglobulin-G-horseradish peroxidase conjugates (Pierce, Rockford, IL, USA), and then exposed to X-ray film. Protein bands were detected using an enhanced chemiluminescence Western blotting detection kit (Amersham, Little Chalfont, USA).

Statistical Analysis

All measurements were made in triplicate and all values were represented as means ± S.E. The results were subjected to an analysis of the variance (ANOVA) using the Tukey test to analyze the difference. p < 0.05 were considered significantly.

RESULT

The radical scavenging effect of triphloretol-A on the ROS generated by γ-ray radiation was measured. Triphloretol-A at 30 μM decreased the level of intracellular ROS induced by radiation (Fig. 2A and B). The level of ROS detected using spectrofluorometer (Fig. 2A) showed 72% of ROS level in triphloretol-A treated irradiated cells compared to 122% of ROS level in irradiated cells. And the level of ROS detected using flow cytometry (Fig. 2B) showed 107 value of fluorescence intensity which was produced from ROS stained by DCF-DA fluorescence dye in triphloretol-A treated irradiated cells compared to 150 value of fluorescence intensity which was produced from ROS level in triphloretol-A treated irradiated cells. The level of ROS generated by triphloretol-A on the ROS level in triphloretol-A treated irradiated cells compared to 122% of ROS level in irradiated cells. And the level of ROS detected using flow cytometry (Fig. 2B) showed 107 value of fluorescence intensity which was produced from ROS stained by DCF-DA fluorescence dye in triphloretol-A treated irradiated cells compared to 150 value of fluorescence intensity in irradiated cells. The abilities of triphloretol-A to inhibit membrane lipid peroxidation and cellular DNA damage in irradiated cells were also investigated. Ionizing radiation induced damage to cell membrane and this one of the most important lesions was responsible for loss of viability. The peroxidation of membrane lipids is the major lesion in the membranes. As shown in Fig. 3, V79-4 cells exposed to γ-rays in vitro showed an increase in the lipid peroxidation, which was monitored by the generation of thiobarbituric acid reactive substance (TBARS). However, triphloretol-A prevented the radiation-induced peroxidation of lipids. Damage to cellular DNA induced by γ-radiation exposure was detected using an alkaline comet assay. The exposure of cells to radiation increased comet parameters like tail length and percentage of DNA in the tails of cells. Treatment with triphloretol-A before irradiation resulted in a decrease of tail length (Fig. 4A). When the cells were exposed to γ-radiation at 10 Gy, the percent of DNA in the tail was increased 50.1 ± 3.4 as shown in Fig. 4B. Treatment with triphloretol-A resulted in a decrease to 29.5 ± 2.3, which indicated a protective effect of triphloretol-A on radiation-induced DNA damage in vitro. The protective effect of triphloretol-A on cell survival in V79-4 cells exposed to radiation was also measured. As demonstrated in Fig. 5A, treatment with triphloretol-A at 30 μM increased 74% of the cell survival compared to 54% of the cell survival in irradiated cells at 10 Gy. This cytoprotective effect by triphloretol-A also showed at radiation-dose dependent manner (Fig. 5B) and at concentration of triphloretol-A dependent manner (Fig. 5C). In order to study the cytoprotective effect of triphloretol-A on radiation-induced apoptosis, nuclei of V79-4 cells were stained with Hoechst 33342 for microscopy and with propidium iodide for flow cytometric analysis. The microscopic pictures in Fig. 5D demonstrated that the control cells had intact nuclei, and the radiation-exposed cells demonstrated significant nuclear fragmentation, which is characteristic of apoptosis. However, when the cells were treated with triphloretol-A for 1 h prior to radiation, dramatic decrease in nuclear fragmentation was observed. In addition to the morphological evaluation, the protective effect of triphloretol-A against apoptosis was confirmed by flow cytometry. As demonstrated in Fig. 5E, an analysis of the DNA content in the radiation-exposed cells revealed an increase of 35% in apoptotic sub-G1 DNA content, compared to 1% of apoptotic sub-G1 DNA content in untreated cells. Treatment with 30 μM of triphloretol-A decreased the apoptotic sub-G1 DNA content to 18%. These results suggested that triphloretol-A protects cell viability by inhibiting radiation-induced apoptosis. To understand how triphloretol-A protects radiation-induced apoptosis, we examined changes of Bcl-2 families and caspases activities by western blot. As shown in Fig. 5F, the Bcl-2, an anti-apoptotic protein, recovered in treatment with triphloretol-A and radiation compared to irradiated cells and the Bax, a pro-apoptotic protein, decreased in treatment with triphloretol-A and radiation compared to irradiated cells. It was also observed the decreased activation of caspase 3 (17 kDa), the major effector caspase of the apoptotic process, and PARP cleavage (89 kDa), one of substrates of activated caspase 3, in combination with triphloretol-A and radiation compared to irradiated cells. Treatment of triphloretol-A at 30 μM after irradiation at 10 Gy still showed the protective effect, increasing 77% of the cell survival compared to 58% of the cell survival in irradiated cells (Fig. 6A). In addition, triphloretol-A reduced intracellular ROS induced by post-irradiation (Fig. 6B).
Exposure to ionizing radiation causes cell damage through the production of ROS which induces oxidative stress. The role of ROS in radiation injury and the potential of antioxidants to reduce these deleterious effects have been studied for more than 50 years. Radioprotective agents minimize this damage by reducing the generation of free radicals. Increased understandings of the interrelationship between the effects of oxygen and radiation exposure lead to a rational application of naturally occurring antioxidants. Our study demonstrated that in addition to an antioxidant effect, triphlorethol-A had protective effect against γ-radiation in vitro. Triphlorethol-A reduced the ROS, which only hydrogen peroxide was detected in this study, generated by radiation. Radiation-induced cellular lethality occurs through the formation of aqueous free radicals, which attack vital cellular sites, such as DNA and cell membranes. The cellular membrane and DNA are the two main targets of radiation-induced lethality. The formation of lipid peroxides in cells exposed to γ-radiation is one of the markers of the membrane damage. Triphlorethol-A protected the cell membrane lipids from the peroxidation damage induced by radiation. In addition, triphlorethol-A inhibited the increase of radiation-induced comet parameters, which indicated the protection of cellular DNA. These inhibitory effects of triphlorethol-A against lipid and DNA damage resulted in protective effects against radiation-induced cell death. Triphlorethol-A is polymer of phloroglucinol and has a polyphenol structure. The presence of a phenolic group with aromatic conjugation, which exists in the structure of triphlorethol-A, contributes to the scavenging ROS generated by irradiation. Triphlorethol-A increased cell survival via inhibition of radiation-induced apoptosis. This cytoprotective effect induced by triphlorethol-A was associated with the increased bcl-2 expression, the decreased bax expression and caspase 3 activity. Our study demonstrated that triphlorethol-A has a radioprotective effect through scavenging ROS generated by radiation.

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