Protective effect of dieckol on γ-ray radiation-induced V79-4 lung fibroblast cell damage involved in modulation of reactive oxygen species

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Abstract

Ionizing radiation can induce oxidative stress through generation of reactive oxygen species (ROS) resulting in cell damage and cell death. We have investigated the radioprotective effect of dieckol, which was isolated from *Ecklonia cava*, against oxidative stress induced cell damage in Chinese hamster lung fibroblast (V79-4) cells. Dieckol was found to reduce the intracellular ROS generated by γ-ray radiation. Moreover, dieckol also protected the cell viability damaged by the radiation through inhibition of apoptosis. Irradiated cells with dieckol treatment reduced the expression of phospho histone H2A.X (a marker for DNA strand breakage) and the activation of caspase 9, which were induced by radiation. These results suggest that dieckol protected γ-ray radiation induced apoptosis of V79-4 lung fibroblast cells by inhibiting ROS generation. (J Med Life Sci 2009;6:368-372)

Key Words : Dieckol, Reactive oxygen species, Cell damage, Apoptosis.

Introduction

Reactive oxygen species, including the superoxide anion, hydroxyl radical, single oxygen, and hydrogen peroxide, are oxygen containing molecules with unpaired electrons or abstract electrons from other molecules. These reactive oxygen species can lead to functional damage in lipid, proteins and DNA, which can eventually result in cell death[1]. Gamma-ray radiation is known to induce oxidative stress via the generation of reactive oxygen species in cells[2, 3]. In many cases, radiation-induced cell death has been identified as apoptosis[4-6].

*Ecklonia cava*is a brown alga (Laminariaceae) that is abundant in the subtidal regions of Jeju island, Korea. It has been reported that the Ecklonia species exhibits radical scavenging activity[7-9], cytoprotective properties against oxidative stress[10-14]. Phlorotannin components of *E. cava* include phenolic secondary metabolites such as eckol (a closed-chain trimer of phloroglucinol), 6,6-bieckol (a hexamer), dieckol (ahexamer), phlorofucofuroeckol (a pentamer) and triphlorethol-A that are influential for biological activities[10, 11, 15]. Among these phlorotannins, dieckol is one of the major and active compounds. Among these phlorotannins, dieckol is one of the major and active compounds. Its attributes include antioxidant activity[15], antiallergic activity[16], inhibition of human immunodeficiency virus-1 reverse transcriptase[17].

This study focused on evaluating the protective effect of dieckol on γ-ray radiation-induced V79-4 lung fibroblast cell damage and cell death involved in ROS.

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This research was performed under the program of Basic Atomic Energy Research Institute (BAERI) which is part of the Nuclear R&D Programs and in part fromthe study of the DNA repair regulation with the disease program [M1063901] funded by the Ministry of Science & Technology of Korea (KOSEF).

Materials and methods

1. Reagents

Dieckol (Fig. 1) was obtained from Professor Nam Ho Lee of Jeju National University, Korea. The purity of dieckol was assessed by HPLC and was > 90%. Dieckol was freshly dissolved in dimethyl sulfoxide (DMSO), yielding a final concentration, which did not exceed 0.1%. 2′, 7′-dichlorodihydrofluorescein diacetate (DCF-DA), [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] bromide (MTT) and Hoechst 33342 were purchased from the Sigma Chemical Company (St. Louis, MO, USA). The primary caspase 9 and anti-phospho histone H2A.X antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA).

2. Cell culture and irradiation

Chinese hamster lung fibroblasts (V79-4) cells from the American Type Culture Collection (Rockville, MD, USA) were maintained at 37℃ in an incubator with a humidified atmosphere of 5% CO_2 and cultured in Dulbecco's modified Eagle's medium, containing 10% heat-inactivated fetal calf serum, streptomycin (100 ㎍/ml) and penicillin (100 units/ml). The cells were exposed to γ-ray radation at 1.5 Gy/min from a ^{60}Co γ-ray source (MDS Nordion C-188 standard source, Jeju National University, Jeju, Korea).

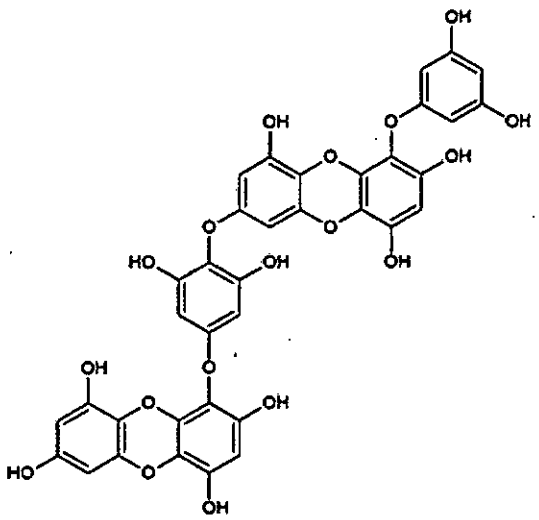
3. Intracellular reactive oxygen species (ROS) measurement

The V79-4 cells were treated with dieckol at 10 ㎍/ml and were exposed to γ-ray radiation an hour later. The cells were incubated for an additional 24 h at 37℃. After adding 25 ㎛ of DCF-DA solution, the fluorescence of 2′, 7′-dichlorofluorescein was detected using a Perkin Elmer LS-5B spectrofluorometer[18].

4. Cell viability

The effect of dieckolon 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[19]. The V79-4 cells were treated with dieckol at 10 ㎍/ml and with γ-ray. Forty eight hours later, 50 ㎕ of the MTT stock solution (2 mg/ml) was added to each well to reach a total reaction volume of 200 ㎕. After incubating for 4 h, the plate was centrifuged at 800 × g for 5 min followed by aspiration of the supernatants. The formazan crystals in each well were dissolved in 150 ㎕ of DMSO and the A_{540} was read on a scanning multi-well spectrophotometer.

Figure 1. Chemical structure of dieckol.



5. Nuclear staining with Hoechst 33342

The V79-4 cells were treated with dieckol at 10 ㎍/ml and with γ-ray radiation at 10 Gy an hour later. Next, the cells were incubated for an additional 48 h at 37℃. 1.5 ㎕ of Hoechst 33342 (stock 10 mg/ml), which is a DNA-specific fluorescent dye, was added to each well and incubated for 10 min at 37℃. The stained cells were visualized under a fluorescent microscope, equipped with a CoolSNAP-Pro color digital camera to examine the degree of nuclear condensation.

6. Western blot analysis

The V79-4 cells were treated with dieckol at 10 ㎍/ml and with γ-ray radiation at 10 Gy, an hour later. Next, the cells were incubated for 48 h at 37℃, and harvested, followed by washing twice with PBS. The harvested cells were then lysed on ice for 30 min in 100 ㎕ 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. The supernatants were collected from the lysates and the protein concentrations were determined. Aliquots of the lysates (40 ㎍ of protein) were boiled for 5 min and electrophoresed in 10% SDS-polyacrylamide gel. The blots in the gels were transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA), and subsequently incubated with anti-primary antibodies. The membranes were further incubated with secondary antiimmunoglobulin-G-horseradish peroxidase conjugates (Pierce, Rockford, IL, USA), followed by exposure to X-ray film. The protein bands were detected using an enhanced chemiluminescence western blotting detection kit (Amersham, Little Chalfont, Buckinghamshire, UK).

7. Statistical analysis

All measurements were made in triplicate and all values were expressed as the means ± standard error of the mean (S.E.M.). The results were subjected to an analysis of variance (ANOVA) using the Tukey test to analyze the difference. $P < 0.05$ was considered significantly.

Results

1. Protective effect of dieckol on γ-ray radiation induced ROS generation

ROS play an important role in irradiation-induced cell

damage. To determine whether the radioprotective effect of dieckol on V79-4 lung fibroblast cells involve in ROS, intracellular ROS were detected by spectrofluorometer. The levels of intracellular ROS increased markedly in V79-4 cells exposure to different dose of irradiation from 0 to 20 Gy (data not shown). We measured the radical scavenging effect of dieckol on the ROS generated by γ-ray radiation at 24 h and found that the level of ROS produced by radiation is increased to 137% compared to control and in dieckoltreated irradiated cells the ROS level is decreased to 119%, suggesting that dieckol scavenged the ROS generated by irradiation (Fig. 2).

Figure 2. Effect of dieckol on scavenging intracellular reactive oxygen species generated by γ-ray irradiation. The V79-4 cells were treated with dieckol at 10 μg/ml, followed by γ-ray irradiation at 20 Gy an hour later. Next, the cells were incubated for 24 h, the intracellular reactive oxygen species was detected using fluorescence spectrophotometer after DCF-DA staining. *Significantly different from 20 Gy irradiated cells ($P < 0.05$).

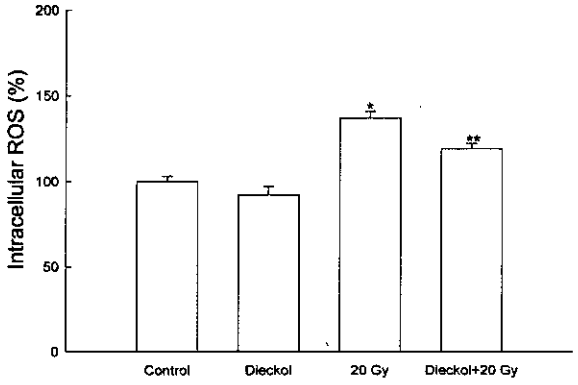
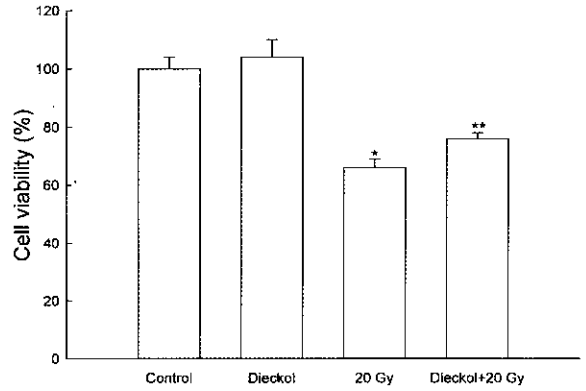


Figure 3. Effect of dieckol on γ-ray irradiation-induced cell death of V79-4 cells. The V79-4 cells were treated with dieckol at 10 μg/ml, followed by γ-ray irradiation at 20 Gy an hour later. Next, the cells were incubated for 48 h. The viability of V79-4 cells on the irradiation was determined by MTT assay. The measurements were made in triplicate and values are expressed as means ± S.E.M. *Significantly different from 20 Gy irradiated cells ($P < 0.05$).



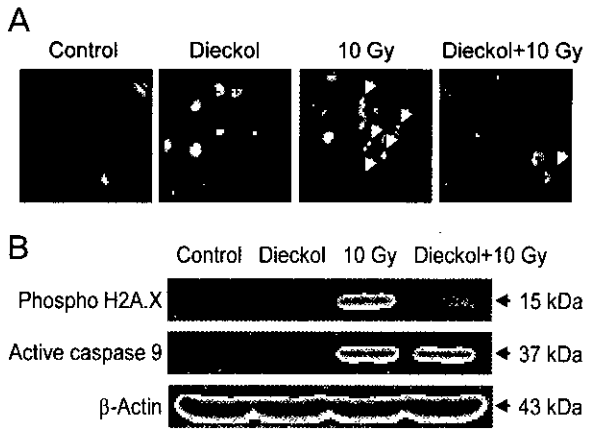
2. Protective effect of dieckol on γ-ray radiation induced cell death

The viabilities of V79-4 lung fibroblast cells exposure to different dose of irradiation were detected to figure out the destructive effect of irradiation. It shown that irradiation reduced cell viabilities in a dose-dependent manner from 0 to 20 Gy (data not shown). The protective effect of dieckol on cell survival in irradiated γ-raycells was measured. Cells were treated with dieckol at 10 g/ml for 1 h, prior to the exposed to radiation. Cell viability was determined 48 h later by the MTT assay. As shown in Fig. 3, treatment with dieckol increased the cell survival by 76% as compared to 66% of irradiated γ-ray at 20 Gy.

3. Protective effect of dieckol on γ-ray radiation induced apoptosis

To evaluate a cytoprotective effect of dieckolon apoptosis induced by γ-ray radiation, the nuclei of V79-4 cells were stained with Hoechst 33342 and assessed by microscopy. The microscopic pictures in Fig. 4A showed that the control cells had intact nuclei, while irradiated γ-ray cellsshowed significant nuclear fragmentation, which is characteristic of apoptosis. However, when the cells were treated with dieckol for 1 h prior to radiation, a dramatic decrease in nuclear fragmentation was observed. In addition, the phosphorylation of the nuclear histone H2A.X, a sensitive

Figure 4. Effect of dieckol upon the γ-ray radiation-induced cellular damage of V79-4 cells. The V79-4 cells were treated with dieckol at 10 μg/ml, followed by γ-ray irradiation at 10 Gy an hour later. Next, the cells were incubated for 24 h. (A) Apoptotic body formation was observed under a fluorescence microscope after Hoechst 33342 staining and apoptotic bodies are indicated by arrows. (B) The cell lysates were electrophoresed, phospho histone H2A.X protein and caspase 9 were detected by a specific antibody.



marker for breaks of double stranded DNA[20], increased in the irradiated γ-ray cells, as shown by western blot (Fig. 4B). However, dieckol treatment in irradiated γ-ray cells decreased the expression of phosphor H2A.X. Next, we examined the activity of caspase 9 by western blot, since caspase 9 is known as an initiator of apoptosis[21]. Dieckol inhibited the γ-ray radiation-induced active form of caspase 9 (37 kDa) (Fig. 4B). These results suggest that dieckol protects cell viability by inhibiting the damage of cellular components and apoptosis induced by γ-ray radiation.

Discussion

Exposure of cells to ionizing radiation can lead to increased generation of ROS, including hydroxyl radicals ((HO•), superoxide anions (O_2^-), singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2), which are major determinants of cellular damage. Therefore, ROS-scavenging agents are considered as radioprotectors[22, 23]. Dieckol is a polymer of phloroglucinol with a polyphenol structure. The existence of a phenolic group with an aromatic conjugation of the structure of dieckol contributes to the quenching of reactive oxygen species generated by irradiation. Our data demonstrate that dieckol produces a radioprotective effect on V79-4 lung fibroblast cells, which are known to be sensitive to irradiation. The protective mechanism of dieckol involves its ability to scavenge ROS. In many cases, the γ-ray radiation-induced cell death has resulted in apoptosis[6, 24]. Dieckol increased cell survival via inhibition of γ-ray radiation-induced apoptosis. This cytoprotective effect induced by dieckol was associated with the inhibited expression of phospho-H2A.X and caspase 9 activity. Taken together, the radioprotective effect of dieckol against γ-ray radiation-induced cell damage was exerted via ROS scavenging activity.

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