Thesis for the Degree of Master of Science

Induction of apoptosis on human hepatocellular carcinoma by dieckol isolated from *Ecklonia stolonifera*

by
Jin Soo Yoon

Department of Microbiology
The Graduate School
Pukyong National University

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(곰피에서 분리된 다이에콜의 인간 간암세포에서 자가세포사멸 유도)

Advisor: Prof. Gun Do Kim

by

Jin Soo Yoon

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Approved by:

(Chairman) Tae Jin Choi

(Member) Young Hwan Song

(Member) Gun Do Kim

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ABSTRACT

Marine algal polyphenols, phlorotannins, which are only known in brown algae. Phlorotannins have been reported to have several biological activities, such as antioxidation, antidiabetic complications, antiamnesia, and chemoprevention against several vascular diseases. In present study, we are targeting on the apoptosis induction capability of dieckol, a phlorotannins isolated from *Ecklonia stolonifera*, on hepatocellular carcinoma Hep3B cells. Dieckol reduced the viable numbers and increased the numbers of apoptotic Hep3B cells in a dose-dependent manner. Western blot analysis revealed that dieckol increased the protein levels of cleaved caspases 3, 7, 8, 9 and cleaved poly (ADP-ribose) polymerase (PARP). Dieckol increased mitochondrial membrane permeability and release of cytochrome c from mitochondria to cytosol with apoptosis inducing factor (AIF). Also, dieckol induces increased expression of truncated Bid and Bim. These results indicate that dieckol induces apoptosis via the activation of both death receptor and mitochondrial-dependent pathways in hepatocellular carcinoma Hep3B cells.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the third leading cause of cancer death with an estimated worldwide incidence of over one million new cases per year [1]. And HCC is a widely prevalent disease in Asia and Africa [2]. Despite extensive exploration for novel anticancer drugs and therapeutic strategies, there has been little success in improving the treatment of HCC. Only surgery offers a cure, but tumor resection is feasible for <15% of patients, and recurrence rates remain as high as 50% after tumor resection due to the aggressive features of HCC including rapid growth, resistance to chemotherapy, and lack of effective adjunct therapy after surgery [3-6]. No effective treatment is available for this carcinoma which is important role in etiology related with Hepatitis B and C [7]. Therefore, we chose dieckol to analyze the effects against hepatocellular carcinoma.

Brown algae are very popular foods, and many people ingest them as a healthy food in Korea and Japan. Even though many studies have been performed on polyphenolic antioxidants derived from terrestrial plants, very limited information has been available for such compounds from marine plants [8]. Marine algal polyphenols, phlorotannins, which are only known in brown algae, are restricted to polymers of phloroglucinol (1,3,5-trihydroxybenzene) [9]. In the previous studies, extracts of several Ecklonia species containing abundant phlorotannins have been reported to possess a number of important biological activities, such as antiplasmin inhibiting activity, HIV-1 reverse transcriptase, protease inhibiting activity, antioxidation, antidiabetic complications, antiamnesia and tyrosinase

inhibitory activity [10-16]. In addition, the isolated phlorotannins from *E. cava* inhibited phosphorylation of JNK and p38 MAPK in human osteosarcoma cell [17]. Moreover the isolated eckol and dieckol from *E. stolonifera* inhibited both NF-kB and MMP-1 expression in human dermal fibroblasts and provided a possibility to develop as an agent for the prevention and treatment of skin aging [18].

Recently, several phlorotannins were isolated from brown macroalgae [10]. The EtOAc-soluble fraction of the E. stolonifera led to the isolation of three phlorotannins [8]. In our study, we are targeting on the apoptosis induction capability of dieckol in those phlorotannins isolated from *E. stolonifera*. The structure of dieckol (Fig. 1.) was identified by comparison with published spectral data as dieckol [19].

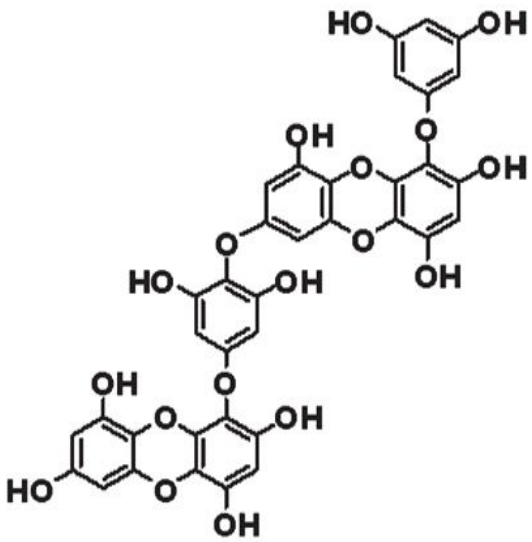

Figure 1. Structure of dieckol isolated from *E. stolonifera* [8].

Apoptosis is a major form of cell death characterized by a series of tightly regulated processes that involved in the activation of a cascade of molecular events leading to cell death. Cells undergoing apoptosis have been found to have an elevated level of cytochrome c in the cytosol and a corresponding decrease in the mitochondria [20]. After the release of mitochondrial cytochrome c, caspase-3 is activated [21], thereby becoming responsible for the proteolytic degradation of PARP, which occurs at the onset of apoptosis [22,23]. Many studies have reported that loss of control of apoptosis results in cancer initiation and progression [24-26]. However, there is no research on dieckol induced cell death in any of the cancer cell lines. We studied the effects of dieckol on HCC to enlighten the cell death pathways.

To the best of our knowledge, the effects of dieckol on HCC and its mechanism of action have yet to be clearly elucidated. The present study was conducted in an effect to determine whether dieckol induces apoptosis in hepatocellular carcinoma cells and to determine the mechanisms underlying this effect. In this study, our aim is to investigate the apoptosis induction and cytotoxic effects of dieckol in hepatic cancer cell line, Hep3B.

MATERIALS AND METHODS

Plant Material.

E. stolonifera was collected along the coast of Busan, Korea, in August 2007. The samples were rinsed using tap water to remove salt. Samples were air-dried under shade for 2 weeks and ground with a hammer grinder, and the dried powder was stored at room temperature until used.

Materials.

The following reagents were purchased from the indicated suppliers: Benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethylketone (Z-VAD-fmk) from TOCRIS bioscience (Ellisville, MO, USA); Dimethyl sulfoxide (DMSO), 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 20,70-dichlorofluorescein diacetate (DCFH-DA), Bovine serum albumin (BSA) standard solution and Propidium iodide from Sigma Aldrich (St. Louis, MO, USA); All solvents were of high-performance liquid chromatography (HPLC) grade and purchased from Fisher Scientific (Pittsburgh, PA); EZ-Cytox Cell Viability Assay Solution WST-1 from Daeil Lab Service (Jong-No, Korea); 4, 6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI) from Roche (Mannheim, Germany); Lysis buffer (50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM DTT, 0.5% NP-40, 1% Triton X-100, 1% Deoxycholate, 0.1% SDS and proteinase inhibitors (PMSF, EDTA, Aprotinin, Leupeptin, Prostatin A) from Intron biotechnology (Gyeonggi, Korea); Protein Quantification Kit (CBB solution®) from Dojindo Molecular Technologies (Rockvile, MD, USA); Nitrocellulose membrane from PALL Life Sciences (MI,

USA); Enhanced chemiluminescent (ECL) detection solutions from Pierce (IL, USA).

Antibodies against Bim and AIF were purchased from Santa Cruz Biotechnology, Inc (CA, USA). Cytochrome c, Bid, Bak, β-actin, cleaved caspase-8, caspase-12, cleaved caspase-3, cleaved caspase-7, cleaved caspase-9, caspase-9, cleaved PARP, HRP conjugated anti-rabbit and anti-mouse antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA).

Extraction and Isolation of Phlorotannins.

The dried powder (4 kg) of *E. stolonifera* was refluxed with MeOH (3 × 9 L) for 3 h. The extract (1.1 kg) was suspended in water (1 L) and partitioned with dichloromethane, ethyl acetate (EtOAc), n-butanol, and solvents in sequence, yielding the dichloromethane (114.8 g), EtOAc (314.7 g), n-butanol (141.5 g), and water (528.2 g) fractions. The EtOAc fraction, which exhibited the most potent antioxidant activity on DPPH radical scavenging activity, was dissolved in dichloromethane and applied to a silica gel (70-230 mesh, Merck) column (100 cm × 10 cm) and eluted with a stepwise mixture of dichloromethane and methanol (6:1, 5:2, 4:3, 3:4, 2:5, 1:6, v/v, each 1.2 L). The eluates were pooled into 17 fractions based on silica gel thin-layer chromatography (TLC) (250 μm, silica gel GF Uniplate, Analtech, Inc., Newark, DE). The TLC plates were developed in a dichloromethane/methanol/water (65:35:10, v/v/v) solvent system. Fractions 7-9 showed high antioxidant activity on DPPH radical scavenging activities, were pooled, and were dried (25 g). The dried sample was dissolved in dichloromethane and applied on the second silica gel column (100

cm × 5 cm) to enhance the antioxidant activity. The column was eluted with a mixture of dichloromethane and methanol (6:1, 5:2, 4:3, 3:4, 2:5, 1:6, v/v, each 0.5 L) and separated into nine subfractions (Fr.1-Fr.9). Fraction 4 (5.80 g), which showed the highest antioxidant activity, was subjected to preparative size exclusion column of Asahipak GS-310 (500 mm × 20 mm, Showa Denko, Tokyo, Japan). An exclusion HPLC apparatus consisted of a pump (Shimadzu LC-6AD), a photodiode array detector (Shimadzu SPDM20A), an online degasser (Shimadzu DUG-$20A_3$), an autosampler (SIL-20A), a fraction collector (Shimadzu FRC-10A), a system controller (CBM-20A), and a Shimadzu LCsolution (ver. 1.22sp). Fraction 4 was chromatographed on an Asahipak GS-310 column eluting with methanol at a flow rate of 5.0 mL/min and monitored at 245 nm. The fraction was separated into five fractions (GS1-GS5). The GS3 fraction (1.110 g) showing high antioxidant activity was chromatographed over Shim-pack PREP-ODS (5 μm, 100 Å , 250 mm × 20 mm, Shimadzu Co., Tokyo, Japan). A preparative ODS HPLC system was similar to the exclusion HPLC system except for a binary pump (Shimadzu LC-6AD) and a column oven (35 C, Shimadzu CTO-20A). The separation of GS3 fraction was conducted using a mobile phase of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The elution profile consisted of a linear gradient from 20 to 100% B solvent for 40 min and re-equilibration of the column with 20% B solvent for 10 min. The flow rate was 7.0 mL/min, and detection was performed at 245 nm. The fraction gave eight subfractions (GS3-ODS1-GS3-ODS8). GS3-ODS1 (67 mg), ODS-3 (150 mg), and ODS7 (144 mg) were purified by the same HPLC system with

a Luna RP-18 column [Luna C18(2), 5 μm, 250 mm × 10 mm, Phenomenex] and with the same mobile phase systems at a flow rate of 3.0 mL/min. The isolated fraction such as GS-ODS3 (dieckol) was used in this study.

Spectrometry.

^{1}H and ^{13}C NMR spectra were determined on a JNM ECP-400 spectrometer (JEOL, Japan), using DMSO-d_6 with tetramethylsilane (TMS) as an internal standard. Heteronuclear multiple quantum correlation (HMQC) and heteronuclear multiple bond correlation (HMBC) spectrawere recorded using pulsed field gradients.

Structural Elucidation of Isolated Phlorotannins.

Dieckol. $C_{36}H_{22}O_{18}$ (MW = 742). ^{1}H NMR (400 MHz, CD_3OD) δ: 6.15 (1H, s, H-300), 6.13 (1H, s, H-3''), 6.09 (2H, s, H-2'', 6''), 6.06 (1H, d, J = 2.9 Hz, H-8), 6.05 (1H, d, J = 2.9 Hz, H-6''), 5.98 (1H, d, J=2.8 Hz, H-6), 5.95 (1H, d, J=2.8 Hz, H-6), 5.92 (3H, s, H-2', 4', 6'). 13C NMR (100 MHz, CD_3OD) δ: 162.7 (C-1'), 161.0 (C-3', 5'), 158.6 (C-1'''), 156.8 (C-7), 155.3 (C-7''), 153.2 (C-3''', 5'''), 148.1 (C-2''), 148.01 (C-2), 147.9 (C-9''), 147.7 (C-9), 145.1 (C-5a''), 145.0 (C-5a), 144.2 (C-4''), 144.1 (C-4'''), 139.4 (C-10a), 139.3 (C-10a''), 127.3 (C-4'''), 127.0 (C-9a), 126.5 (C-1), 126.4 (C-1''), 125.7 (C-9a''), 125.5 (C-4a''), 125.4 (C-4a), 100.7 (C-8''), 100.6 (C-8), 100.3 (C-3), 100.2 (C-3''), 98.5 (C-40), 97.0 (C-2''', 6'''), 96.7 (C-6''), 96.6 (C-6'), 96.2 (C-2', 6').

Cell culture.

Human hepatocellular carcinoma Hep3B cells were obtained from the American Tissue Culture Collection (ATCC, Rockville, MD, USA). Hep3B cells were cultured in Minimum Essential Medium with Earle's Balanced Salts (MEM/EBSS) (HyClone, Logan, UT, USA), 10% heat inactivated fetal bovine serum (HyClone), 100 U/ml penicillin and 100 ㎍/ml streptomycin (PAA Laboratories GmbH, PA, Austria) at 37℃ in a humidified atmosphere of 5% CO_2.

Cell viability assay.

Exponential phase of Hep3B cells at a density 1 x 10^4 cells were resuspended in 100 ㎕ of EMEM medium and seeded on 96-well plate in triplicates. Following overnight incubation, dieckol at various concentrations (80, 100, 120 and 140 ㎛M) was added. Cells were incubated for 24 h, and 10 ㎕ of WST-1 solution was added and incubated for additional 3 h. The absorbance of the reaction was measured using ELISA reader (Molecular Devices, Silicon Valley, CA, USA) at 460 nm and inhibitory rates were calculated. On the other hand, mid-log phase of Hep3B cells were divided into four groups to compare caspase dependent and caspase independent cell death: control, Z-VAD-fmk, dieckol and dieckol with Z-VAD-fmk groups. After passage 24 h, cells were changed to the fresh medium containing none (control group), Z-VAD-fmk (50 ㎛M Z-VAD-fmk), dieckol (120 ㎛M dieckol) and the combination (120 ㎛M dieckol and 50 ㎛M Z-VAD-fmk). Following overnight incubation 10 ㎕ of WST-1 solution was added onto each well and further incubated for 3 h and then readed at 460nm using ELISA reader.

DAPI staining.

To see the formation of apoptosome, Hep3B cells, after incubation an 37 ℃ for 24 h with two different concentrations of 80 and 100 μM dieckol were rinsed once with PBS buffer and stained by the addition of DAPI solution (1 μg/ml) to the plates. After incubation in dark at 37 ℃ for 20min, cells were rinsed once with methanol. The results were examined under an ECLIPSE 50i fluorescence microscope (Nikon, Tokyo, Japan)

Cell cycle analysis for sub-G1 population

Cell cycle distribution was examined using flow cytometry. Briefly, cells were harvested by trypsinisation, fixed in 70% ethanol at 4℃ overnight, and then resuspended in PSB added 0.2 mg/ml (final concentration) RNase A for 1 hour at 37℃. DNA was stained with 40 μg/ml (final concentration) propidium iodide for 30 min. And cells were then examined by FACS Calibur (Becton Dickinson, Mountain View, CA, USA).

Western blot analysis

For Western blotting, the Hep3B cells were cultured as mentioned above and treated with 80 and 100 μM dieckol, and then harvested after 24 h. The harvested cells were collected by centrifugation, lysed in ice-cold lysis buffer. After incubation on ice for 30 min, the insoluble materials were removed by centrifugation at 14,000 rpm for 20 min. The protein content of the cell lysates were determined by a Protein Quantification Kit (CBB solution®) with bovine serum albumen (BSA) as standard. An aliquot from each sample was boiled with sample buffer for 5 min, and then resolved

by 12% SDS-polyacrylamide gel-electrophoresis (SDS-PAGE). The protein were electrotransferred to a nitrocellulose membrane and then blocked in PBST buffer (135 mM Sodium chloride, 2.7 mM Potassium chloride, 4.3 mM Sodium phosphate, 1.4 mM Potassium dihydrogen phosphate, 0.5% Tween-20) containing 5% Skim Milk for overnight at 4℃. The blots were probed with the primary antibodies (anti-cytochrome c, anti-Bid, anti-Bak, anti-Bim, anti-AIF, anti-β-actin, anti-cleaved caspase 8, anti-caspase 12, anti-caspase 12, anti-cleaved caspase 3, anti-cleaved caspase 7, anti-cleaved caspase 9, anti-caspase 9 and anti-cleaved PARP) and then washed three times in PBST, followed by incubation for 1 h with horseradish peroxidase-coupled anti-rabbit IgG or anti-mouse IgG as second antibodies. The blots were then washed in PBST and visualized by an enhanced chemiluminescent (ECL) detection solutions.

RESULTS

Antiproliferation effect of dieckol

HCC derived Hep3B was used and the effect of dieckol treatment on cells was examined by cell viability assay using WST-1 solution. The cell viability was inhibited and dieckol induced cell death in a dose dependent manner. In Hep3B, dieckol (80 - 140 μM) produced a dose dependent reduction in cell growth. Cell death reached 34.5% with 100 μM and 20.9% with 80 μM dieckol treatment for 24 h (Fig. 2) and these concentrations were used in subsequent experiment to investigate the mechanism of cell death. Hep3B cells treated with or without dieckol were visualized under inverted microscope (x 200) (Fig. 3).

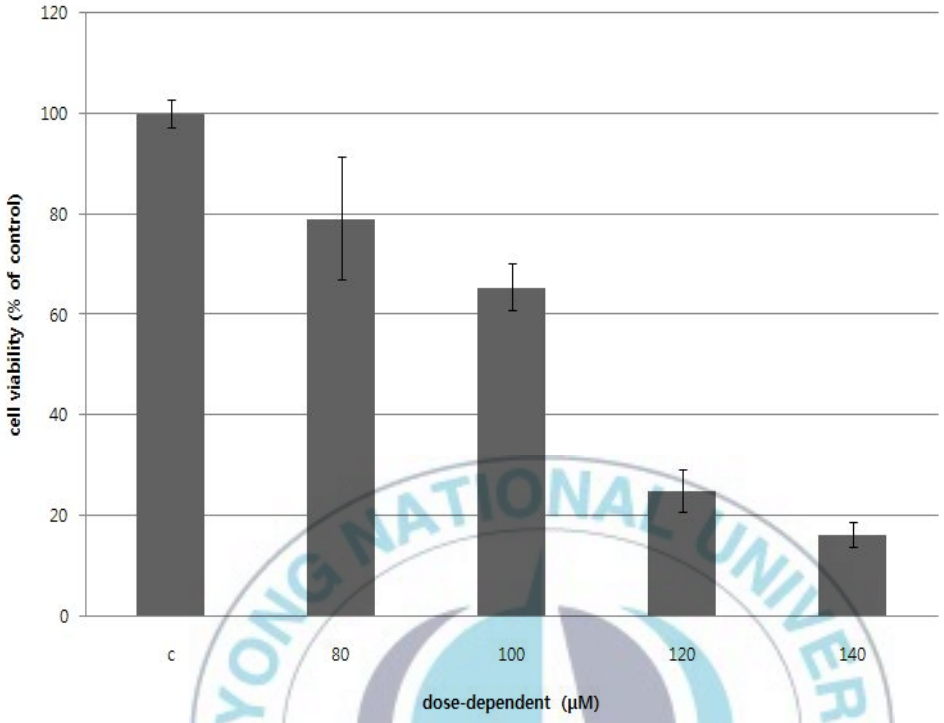

Figure 2. Cell viability determined by WST-1 assay after treatment with 80, 100, 120 and 140 μM dieckol for 24 h.

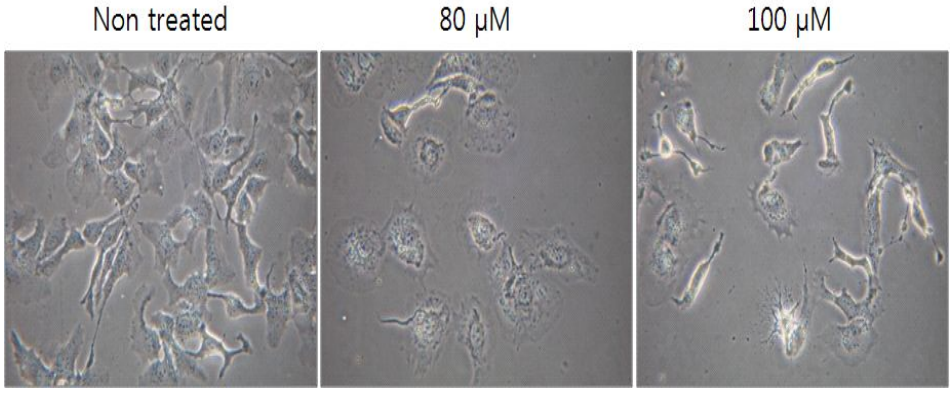

Figure 3. cell population visualized after treatment with 0, 80 and 100 μM dieckol for 24 h by an inverted microscope. Magnification, x 200.

Detection of nuclear membrane changes by DAPI staining

The death of Hep3B cells induced by treatment for 24 h with 80 and 100 μM dieckol showed the signs of apoptosis. To detect nuclear condensation and formation of apoptosome, the cells were stained with DAPI (4', 6-diamidino-2-phenylindole dihydrochloride hydrate) as described above and analyzed under the flourescence microscope (x 1000). The results of DAPI stain showed totally different patterns in the treated and untreated cells. The cells of treated 80 μM dieckol appeared the nuclear membrane changes such as blebbing and shrinkage. A classical hallmark of apoptotic cells, nuclear condensation (apoptosome), appeared in 100 μM dieckol treated Hep3B cells for 24 h, also (Fig. 4).

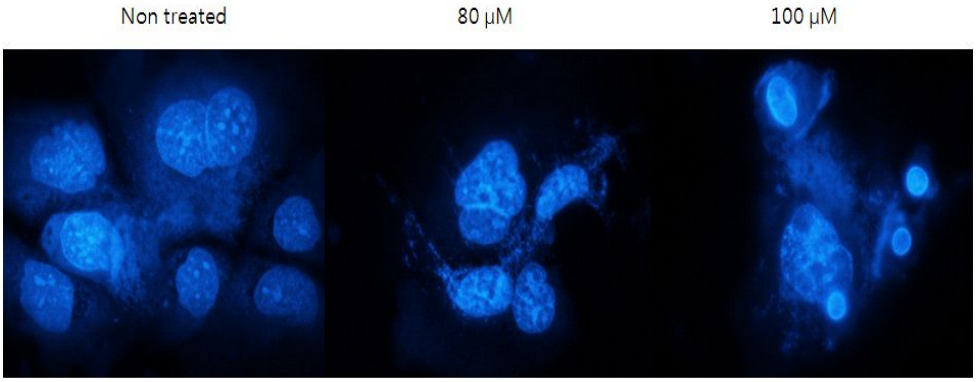

Figure 4. Induction of apoptosis by dieckol on Hep3B cells. DAPI staining, cells contained clearly condensed chromatin or exhibiting fragmented nuclei were taken as apoptotic body. Magnification, x 1000.

Cell cycle analysis for sub-G1 population by Flow cytometry

To investigate the mechanisms of growth inhibition, Hep3B cells were incubated with different doses of dieckol for 24 h. The cells in apoptosis were then identified with DNA-PI flow cytometry. The apoptotic cells, which had fragmented DNA and were shown as sub-G1 in DNA histogram, were increased from 4.49% at 80 μM to 11.60% of the total population at 100 μM dieckol (Fig. 5). Therefore the proportion of apoptotic cells (sub-G1 fraction) increased in a dose-dependent manner.

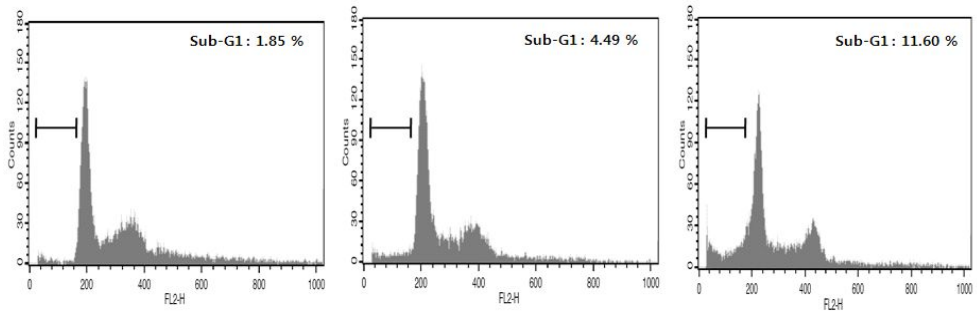

Figure 5. Induction of apoptosis by dieckol on Hep3B cells. FACS analysis for quantification of sub-G1 DNA content of Hep3B cells treated with 80 μM and 100 μM dieckol for 24 h.

To compare caspase-dependent and caspase-independent cell death

The relationship between caspase dependent and caspase independent was examined using dieckol with or without Z-VAD-fmk. Cells were divided into four groups: Control (no treatment), Z-VAD-fmk (treated with 50 μM Z-VAD-fmk), dieckol (treated with 120 μM dieckol) and the combination (treated with both 120 μM dieckol and 50 μM Z-VAD-fmk) (Fig. 6). In Hep3B cells, the dieckol treated resulted in the death of 75% of the cells, about a 45.2% in the dieckol and Z-VAD-fmk combination group. These results suggest that dieckol enhances apoptotic cell death in caspase dependent pathway.

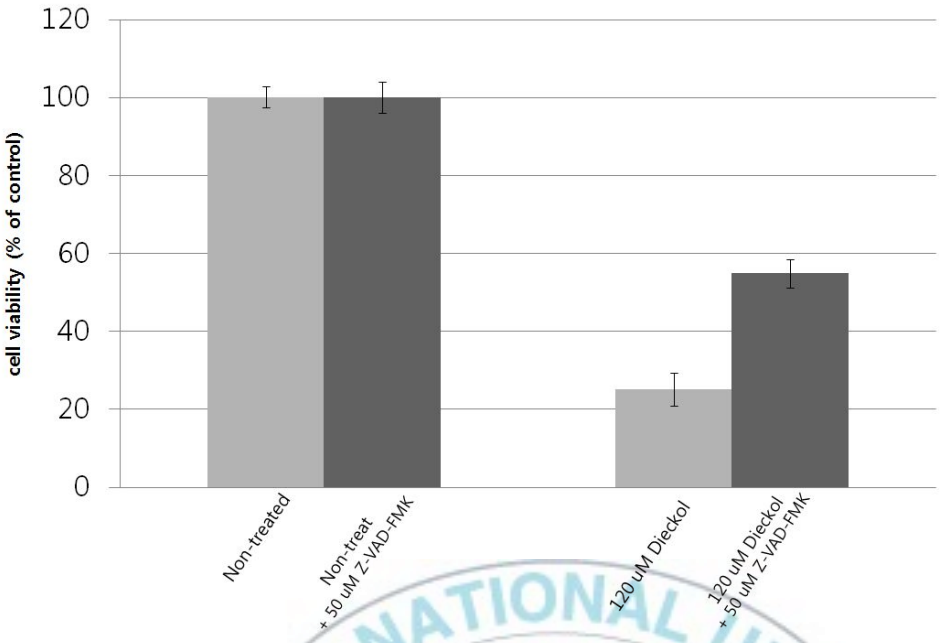

Figure 6. Effect of dieckol on Hep3B cells. Z-VAD-fmk enhances the caspase inhibition in Hep3B cells. Hep3B cells were divided into four groups : control (no treatment), Z-VAD-fmk (treated with 50 μM Z-VAD-fmk), dieckol (treated with 120 μM dieckol) and combination of both (treated with 120 μM dieckol and 50 μM Z-VAD-fmk). The cell viability was measured with WST-1 assay.

The activation of apoptosis-related proteins by Western blot

As shown in Figure 7 and Figure 8, Western blot analysis revealed that dieckol-induced apoptosis events such as the release of cytochrome c. These result included the possible involvement of the dramatically increased expression of Bid and Bim as an initial signal provoking the mitochondrial cytochrome c release. Moreover the result showed that activated form, truncated 57 kDa AIF (Apoptosis Inducing Factor), was gradually increased in a dose-dependent manner (Fig. 7). Consistent with the induction of sub-G1 cell population in flow cytometry, activation of procaspase 9, 12 as well as PARP, as evidenced by the appearance of the cleaved caspase 3, 7, 8, 9 and cleaved PARP products, were observed in Hep3B cells treated with different dieckol dosage for 24 h (Fig. 8).

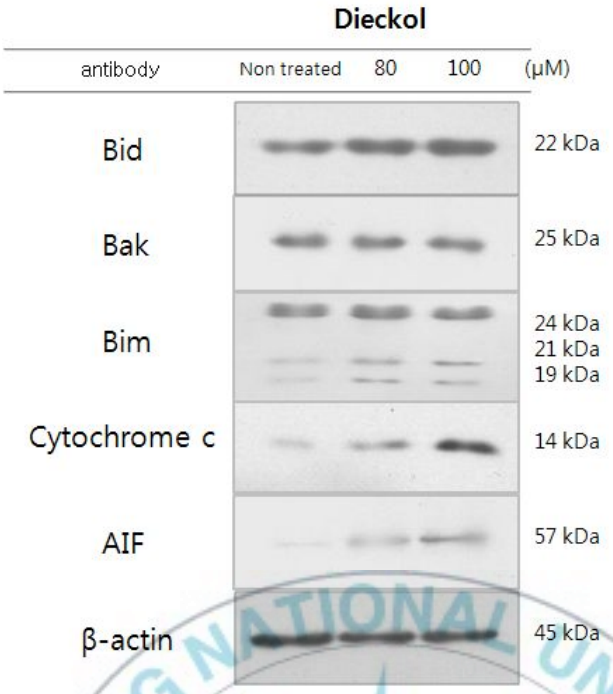

Figure 7. The effects of dieckol for detecting protein levels of apoptosis related genes on Hep3B cells. Cells were treated with 0, 80 and 100 μM dieckol for 24 h. Note that dieckol modulates the ratio between the amounts of BiD, Bak, Bim, cytochrome c, AIF and β-actin. Photographs of chemiluminescent detection of the blots, which were representative of three independent experiments, are shown.

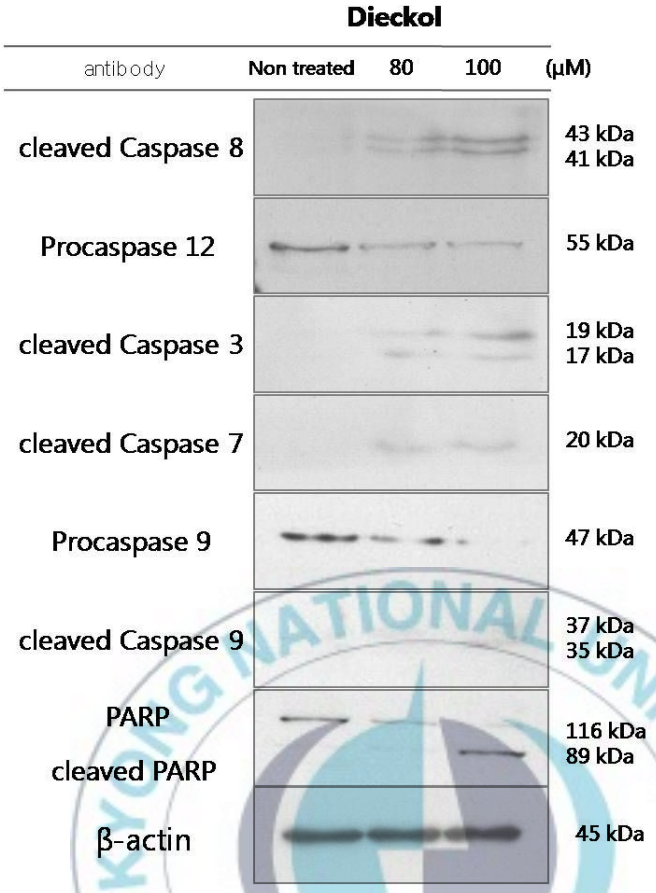

Figure 8. The effects of dieckol for detecting protein levels of apoptosis related genes on Hep3B cells. Dieckol induces activation of the critical molecules (cleaved caspase 3, 7, 8, 9, 12, PARP and β-actin). Cell lysates were analyzed via western blotting with the indicated antibodies. Photographs of chemiluminescent detection of the blots, which were representative of three independent experiments, are shown.

DISCUSSION

In this study, we demonstrated that dieckol induced the expression of Bim and Bid through AIF activation and released cytochrome c, leading to apoptosis in Hep3B. The extrinsic/death receptor pathway is mediated by cell surface death receptors, such as Fas. The binding of death ligands, such as Fas-L, to their specific receptors results in the recruitment of the adaptor protein Fas-associated death domain and caspase-8, leading to the cleavage and activation of caspase-8. Activated caspase-8 amplifies the apoptotic signal either direct activation of downstream executioner caspase-3 or cleavage of Bid (a BH3-only pro-apoptotic Bcl-2 family protein) subsequently leading to the release of cytochrome c [27,28]. We determined that the protein levels of cleaved caspase-8 and Bid were increased in dose dependent manner (Fig. 7). These results indicate that caspase-8 promotes caspase-3 activation directly in dieckol treated cells, rather than through activating caspase-9 (Fig. 8).

Mitochondrial membrane permeability is precisely regulated by the Bcl-2 family proteins [29], are subdivided into three classes on the basis of their functions and the numbers of Bcl-2 homology (BH) domains present: the anti-apoptotic proteins such as Bcl-2, Bcl-xL, and Mcl-1 that harbors four BH domains (BH1- BH4); the pro-apoptotic proteins, including Bax, Bak, and Bok, which harbors three BH domains (BH1- BH3) and the BH3-only pro-apoptotic proteins, including Bik, Bim, Bad, Bmf, and Bid, which shares homology only within the BH3 domain. These proteins are capable of forming either homo-oligomers or heterodimers with one another and appear to perform distinct functions in the regulation of mitochondrial

membrane permeabilization [30]. In the current study, we determined that dieckol increased the protein levels of pro-apoptotic Bim_S, Bim_L and Bid (Fig. 7). Bim consists of three isoform, Bim_S, Bim_L and Bim_{EL} (Bim-short, Bim-long and Bim-extra long, respectively) with different intrinsic toxicities by alternative splicing [31] and all are performed in different modes of regulation by various pro-death and pro-survival signaling pathways [32]. Bim also mediates ER stress signaling for activation of caspase-12 during ER stress-induced apoptosis [33]. Our results shows that the increased expression of pro-apoptotic proteins by dieckol and disruption of mitochondrial membrane permeability promotes cytochrome c release with AIF, thereby activation of caspase-9 and caspase 3 in Hep3B cells.

AIF to the cytosol and to the nucleus was known to be cause of peripheral chromatin condensation and large-scale fragmentation of DNA. In Rat-1 cells, after sufficient release of cytochrome c from the mitochondria to the cytosol, chromatin condensation and nuclear fragmentation (karyorhexis) by AIF were progressed [34]. Moreover, there is a lot of evidence that the mitochondria plays essential role in apoptosis by releaseing apoptogenic factors, such as cytochrome c and AIF from the intermembrane space into the cytoplasm, which activates the downstream executional phase of apoptosis [30]. AIF released from the mitochondria and participate in caspase independent apoptosis [34]. Apoptosis occurs via the coordinated activation of executioner caspases, which cleaved a variety of cellular substrates, including PARP, a nuclear enzyme involved in DNA repair [27]. Therefore, we also investigated the expression of AIF induced apoptosis related genes, cleaved PARP, cleaved caspase-3 and cleaved caspase-7. Cell lysates were

subjected to immunoblotting analysis with antibodies against PARP and the cleaved caspases indicated and the results also showed that the activated form of those genes were increased (Fig. 8). It has been established that these executioner caspases are activated by initiator caspases, including caspase-8 and -9 [27]. Our results show that dieckol induces the activation of caspase-8 and -9, resulting in the activation of caspase-7 and -3, which provokes the induction of apoptosis in hepatocellular carcinoma.

The activation of caspases is triggered principally via two distinct pathways-the extrinsic/death receptor pathway and the intrinsic/mitochondrial pathway. The activation of the intrinsic/mitochondrial pathway is involved in the permeabilization of the outer mitochondrial membrane with subsequent releases of some pro-apoptotic factors, including cytochrome c, into the cytosol. Cytosolic cytochrome c alters the conformation of the cytosolic protein Apaf-1, whereas this protein oligomerizes with inactive procaspases- 9, thus resulting in the activation of this enzyme [35].

The results presented here indicates, for the first time, that dieckol increases mitochondrial membrane depolarization in Hep3B cells by cytochrome c with AIF release from the mitochondria, which, in turn, results in caspase-9 activation, subsequently resulting in the activation of caspase-3 and -7. As summarized in Fig. 9, our results demonstrated that dieckol induced apoptosis which acts through multiple signaling cascades, through the activation of caspases-3, -7, -8, -9 and PARP. Collectively, our results suggest that the activation of the extrinsic/death receptor and intrinsic/mitochondrial pathway contributes to dieckol-induced apoptosis in Hep3B cells.

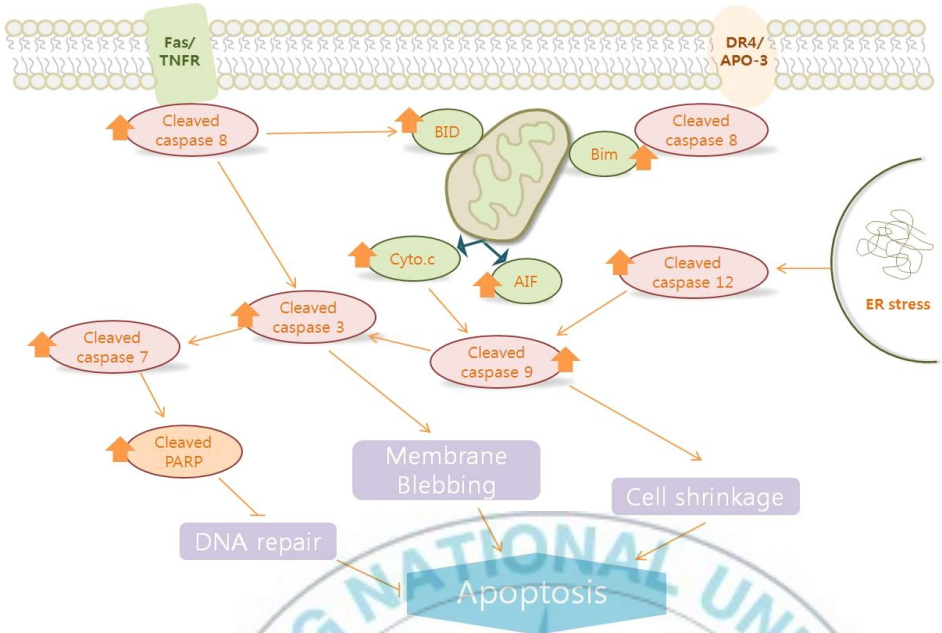

Figure 9. Proposed dieckol-induced apoptosis signaling on Hep3B cells.

국문초록

세포의 죽음은 necrosis와 apoptosis로 구분되며, 이것은 세포의 형태학적 및 생화학적인 특성에 의하여 구분될 수 있다. 그 중 apoptosis는 개체의 발생단계나 DNA 손상, 바이러스 감염 등에 따른 유전적 조절 하에서 일어나는 개체보존 수준에서 손상된 세포들의 제거를 위한 중요한 방어기전이라는 점에서 생리적 또는 화학적 외상에 의한 세포의 죽음인 necrosis와 구별된다. 최근 apoptosis 조절에 관여하는 유전자들의 역할에 관한 지식이 증대되면서 apoptosis 조절의 중요성이 인체 질병과 연관되어 더욱 많은 관심 분야로 인식되어지고 있다. 따라서 apoptosis 유발 해석에 대한 연구는 특히 암세포의 과다한 증식과 성장억제에 대한 연구에 필연적인 것으로 사료된다.

Dieckol은 Brown algae (갈조류) 강 (Phaeophyceae), kelp (미역) 목 (Laminariales), kelp (미역) 과 (Lessoniaceae)에 속하는 *Ecklonia stolonifera*에서 분리된 hexameric compound로 항염증 반응 (anti-inflammatory effect), pro-inflammatory cytokines의 생성 억제, LPS-induced nuclear factor κB (NF-κB)와 p38 mitogen-activated protein kinases (MAPKs) 활성 감소, reactive oxygen species (ROS) 생성 억제, 기억력 증진, 항 고혈압 효과 등이 있는 것으로 알려져 있으나, 아직 정확한 항암 기전은 밝혀지지는 않았다.

본 연구에서는 dieckol이 Hep3B 간암 세포에서 apoptosis를 일으키는 기전을 알아보고자 Western blot 실험을 통하여 cleaved caspase 3, 7, 8, 9과 cleaved PARP 등의 단백질 발현양상을 분석하여 apoptosis를 일으킴을 확인할 수 있었다. 또한 dieckol은 미토콘드리아와 연관된 cytochrome c와 AIF를 방출하면서 하위 caspases를 활성화 시키게 되는데 이는 Bcl family 중 Bid와 Bim 의 과 발현에 의해 일어나는 것으로 확인되었다. 따라서 본 연구는 dieckol이 Hep3B 간암 세포에 일으키는 apoptosis가 death receptor에 의한 세포 외 기작과 미토콘드리아에 의한 세포 내 기작이 동시에 작용하여 일어나는 현상임을 알 수 있었다.

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