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ARTICLE

6-Dehydrogingerdione Sensitizes Human Hepatoblastoma Hep G2 Cells to TRAIL-Induced Apoptosis via Reactive Oxygen Species-Mediated Increase of DR5

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The anticancer effects of 6-dehydrogingerdione (6-DG), a compound isolated from the rhizomes of *Zingiber officinale*, and its mechanisms of sensitization to TRAIL-induced apoptosis were studied using human hepatoblastoma Hep G2 cells. This study demonstrates for the first time that 6-DG-induced apoptosis might be executed via mitochondrial- and Fas receptor-mediated pathways. Further studies also demonstrated that 6-DG could sensitize Hep G2 cells to TRAIL-induced apoptosis. 6-DG also up-regulated Ser-15 phosphorylation and evoked p53 nuclear translocation. Abrogation of p53 expression by p53 small interfering RNA significantly attenuated 6-DG-induced DR5 expression, thus rendering these cells resistant to TRAIL-induced apoptosis. DR5 expression after 6-DG treatment was accompanied by provoking intracellular reactive oxygen species (ROS) generation. Pretreatment with *N*-acetyl-L-cysteine (NAC) attenuated 6-DG-induced DR5 expression and inhibited TRAIL-induced apoptosis. In contrast to Hep G2 cells, DR5 up-regulation and sensitization to TRAIL-induced apoptosis instigated by 6-DG were not observed in normal MDCK cells. Taken together, these data suggested that in addition to the mitochondrial- and Fas receptor-mediated apoptotic pathways involved, ROS-dependent and p53-regulated DR5 expression was also demonstrated to play a pivotal role in the synergistic enhancement of TRAIL-induced apoptosis instigated by 6-DG in Hep G2 cells.

KEYWORDS: Apoptosis; 6-dehydrogingerdione; DR5; reactive oxygen species

INTRODUCTION

TNF-related apoptosis-inducing ligand (TRAIL) is considered to be a promising cancer-specific agent due to its ability to selectively induce apoptosis in a variety of malignant cells with little or no toxicity to nontransformed cells (*1*). However, recent studies have shown that some cancer cells, including hepatoma cells, can acquire resistance to TRAIL-induced apoptosis (*2*, *3*). Hence, new strategies to overcome the resistance of hepatoma cells are urgently needed. Until recently, many studies have demonstrated that TRAIL-resistant cancer cells can be sensitized by chemotherapeutic drugs through the induction of death receptor 5 (DR5) expression (*4*−*7*). Therefore, sensitization to TRAIL-induced apoptosis by the up-regulation of DR5 may underscore a potential strategy for treating TRAIL-resistant hepatoma cells.

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TRAIL cross-links with DR4 or DR5, resulting in DR4 or DR5 trimerization and intracellular death domain clustering, leading to the formation of a death-inducing signaling complex. This complex can then recruit the Fas-associated death domain adaptor molecule and subsequently activate caspase-8 (*8*).

The p53 tumor suppressor gene product is a transcription factor that regulates cellular response to DNA damage (*9*, *10*). Post-translational modification, such as phosphorylation or acetylation of specific amino acids of the p53 protein, has been known to modulate its activity (*11*−*13*). Phosphorylation on NH_2-terminal residues, especially Ser-15, Thr-18, Ser-20, or Ser-37, is believed to inhibit the interaction between p53 and MDM2 and hence contribute to p53-dependent transactivation (*12*). Furthermore, p53 phosphorylation at the residue of Ser-15 has been linked to apoptosis induced by chemotherapeutic and chemopreventive agents (*14*). Recent studies have shown that DR5 can be transactivated by p53 through an intronic sequence-specific p53BS (*15*−*17*).

Chemoprevention by the use of naturally occurring substances is becoming a promising strategy to prevent cancer. Ginger

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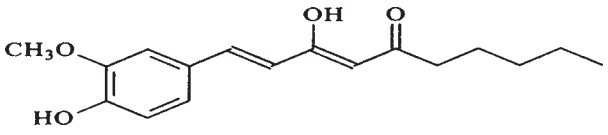

Figure 1. Chemical structure of 6-dehydrogingerdione (6-DG).

(*Zingiber officinale*) is an edible plant and has been widely used as a spice. It contains several pungent constituents that possess anticarcinogenic activities (*18*, *19*). 6-Dehydrogingerdione (6-DG) (**Figure 1**) is a phenolic alkanone component isolated from the rhizomes of ginger (*20*). The aim of this study was to examine its anticarcinogenic effect on human hepatoblastoma Hep G2 cells. In this study, we demonstrate for the first time that 6-DG could trigger apoptotic cell death via both mitochondrial- and Fas receptor-mediated pathways. In addition, we present the first evidence that 6-DG could also up-regulate DR5 expression and synergistically enhanced TRAIL-induced apoptosis in human hepatoblastoma Hep G2 cells.

MATERIALS AND METHODS

Plant Material. The roots of *Zingiber officinale* (ginger) were purchased from a local market in Kaohsiung, Taiwan, in June 2008, and were identified by Dr. Yen-Ray Hsui of the Division of Silviculture, Taiwan Forestry Research Institute, Taipei, Taiwan. A voucher specimen (Hsui-Zo-1) was deposited at Fooyin University.

Extraction and Isolation. The rhizomes (25.6 kg) of *Z. officinale* were chipped and air-dried and extracted repeatedly with $CHCl_3$ (50 L × 4) at room temperature. The combined $CHCl_3$ extracts (896.5 g) were then evaporated and further separated into 20 fractions by column chromatography on silica gel (3.8 kg, 70−230 mesh) with *n*-hexane/$CHCl_3$/MeOH. Fraction 8 (81.2 g), eluted with $CHCl_3$/MeOH (60:1), was next subjected to silica gel CC ($CHCl_3$/MeOH (100:1)) and yielded 6-DG (163 mg). The other fractions were further processed for other components unrelated to this study. The identity and purity of 6-DG were confirmed by NMR and HPLC. Stock solutions of 6-DG (1M) were made by dissolving this compound in DMSO. Various aliquots of diluted preparations were then frozen at −20 °C until use.

Chemicals. Rhodamine 123, propidium iodide, and 2′,7′ -dichlorodihydrofluorescein diacetate (DCFH-DA) utilized herein were acquired from Molecular Probes (Eugene, OR). G418 and protease inhibitor cocktail were from Calbiochem (La Jolla, CA). Soluble recombinant human TRAIL/Apo2L was purchased from R&D systems (Minneapolis, MN). Rabbit polyclonal antibody specific for DR5 was purchased from ProSci (Poway, CA). Antibodies against caspase-3, caspase-8, caspase-9, p53, phospho-p53 (Ser-15), FAS, Fas-L, Bid, Mcl-1, and COX IV were from New England Biolabs. Monoclonal anticytochrome *c* and Fas-L neutralizing antibody (NOK-1) were purchased from BD PharMingen (San Diego, CA). Mouse monoclonal antibody specific for β-actin was from Sigma-Aldrich. Antibodies against Bax and Bcl-2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Lipofectamine 2000 reagent was obtained from Invitrogen (Carlsbad, CA).

Cell Culture. The human hepatoblastoma Hep G2 and normal tubular epithelial Madin−Darby canine kidney (MDCK) cell line were obtained from the American Type Culture Collection (Rockville, MD). These cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 1% penicillin/streptomycin. The cells were grown at 37 °C and 5% CO_2 in a humidified environment.

Apoptosis Assay. Hep G2 cells were grown and treated with vehicle (0.1% DMSO) or various concentrations of 6-DG for 24 h. After drug treatment, cells were subsequently collected and suspended in 30 μL of ice-cold Tris-EDTA (pH 8.0), to which were added 12 volumes of 6 M guanidine-HCl, 1 volume of 7.5 M ammonium acetate, 1 volume of 20% sodium dodecyl sulfate, and 1 volume of proteinase K (3 mg/mL). Lysate was incubated at 50 °C for 2 h, and genomic DNA was extracted using phenol/chloroform/isoamyl alcohol (25:24:1) and precipitated with absolute alcohol. DNA samples were electrophoresed on a 1.8% agarose gel at 100 V for 40 min and visualized with ethidium bromide staining under UV illumination.

Quantitative analysis of apoptotic cells was determined by the assessment of the percentage of hypodiploid DNA (sub-G1). Briefly, after treatment with vehicle (0.1% DMSO) or various concentrations of 6-DG for 24 h, cells were harvested by trypsinization and fixation in PBS/MeOH (1:2, v/v) at 4 °C for at least 18 h. After a wash with PBS, the cell pellets were suspended in PBS (500 μL) and incubated with 2.4 μL of RNase A (10 μg/mL) and the same volume of propidium iodide (10 μg/mL) in the dark for 30 min. The stained cells were analyzed using a Becton-Dickinson FACS-Calibur flow cytometer.

Establishment of p53-Knockdown (Hep G2/p53shRNA) and Nonsilencing Control (Hep G2/SMAD4shRNA) Cell Lines. The template for human p53 shRNA was constructed by ligating the annealed oligonucleotides 5′-GATCCCCGACTCCAGTGGTAATCTACTTCA-AGAGAGTAGATTACCACTGGAGTCTTTTTGGAAC-3′ and 5′-TCGAGTTCCAAAAAGACTCCAGTGGTAATCTACTCTCTTGA-AGTAGATTACCACTGGAGTCGGG-3′ into the *Bgl*II and *Hin*dIII sites of pSUPER. As a nonsilencing control, the oligonucleotides 5′-GAT-CCCCCTGGCATCGGTGTGGATGATTCAAGAGATCATCCAC-ACCGATGCCAGTTTTTGGAAA-3′ and 5′-AGCTTTTCCAAAAA-CTGGCATCGGTGTGGATGATCTCTTGAATCATCCACACCGA-TGCCAGGGG-3′ were used, which were derived from mouse SMAD4 and have been demonstrated to be nonfunctional in mouse and human cells (*21*). To establish stable cell lines, Hep G2 cells were separately transfected with p53shRNA and SMAD4shRNA plasmids using lipofectamine 2000 reagent. Stable cell lines were selected with fresh media containing 1 mg/mL G418. The silencing of p53 in Hep G2/p53shRNA and Hep G2/SMAD4shRNA cells was confirmed by Western blotting.

Determination of ROS Production. Production of intracellular H_2O_2 and/or peroxide was detected by flow cytometry using DCFH-DA (*22*). Hep G2 cells were grown and treated with 6-DG. At the end of each experimental period, cells were incubated with DCFH-DA (10 μM) for 30 min in the dark and resuspended in PBS containing propidium iodide (5 μg/mL) for 10 min prior to flow cytometry, with excitation and emission settings of 488 and 525−550 nm, respectively.

Measurement of Mitochondrial Membrane Potential (MMP) by Flow Cytometry. The MMP was determined by flow cytometry after staining with rhodamine 123 (*23*). Rhodamine 123 is a fluorescent dye that is incorporated into mitochondria in a transmembrane potential-dependent manner. Hep G2 cells were seeded onto a 60 mm tissue culture dish and grown for 24 h. Following treatment with 100 μM 6-DG for 6, 12, and 24 h, cells were stained with 5 μM rhodamine 123 for 30 min in the dark. The MMP was determined by analyzing the fluorescent level of rhodamine 123 using a Becton-Dickinson FACS-Calibur flow cytometer.

Cell Lysates Preparation and Immunoblotting. Total cell lysates were prepared as described previously (*22*). Briefly, control (0.1% DMSO) and 6-DG-treated cells were collected by centrifugation, and then the pellets were resuspended in a lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM phenylmethabesulfonyl fluoride) at 4 °C for 1 h.

Nuclear extracts were isolated as described (*4*). Briefly, cells were pelleted and resuspended in hypotonic buffer (10 mM HEPES (pH 7.5), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM NaF, 1 mM DTT, 1 mM Na_3VO_4, 1 mM phenylmethanesulfonyl fluoride). After incubation on ice for 15 min, 0.5% Nonidet P-40 was added and vigorously vortexed for 15 s. The nuclei were pelleted and resuspended in a buffer (20 mM HEPES (pH 7.5), 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 1 mM DTT, 1 mM Na_3VO_4, 1 mM phenylmethanesulfonyl fluoride) containing a protease inhibitor cocktail. The nuclear extracts were centrifuged, and the supernatants were frozen at −80 °C.

Mitochondrial and cytosolic fractions were prepared as described previously (*22*). For Western blotting, equal amounts of proteins were resolved on 12% polyacrylamide gel and transferred to nitrocellulose membrane. After blocking with 5% nonfat milk for 1 h at room temperature, the membrane was incubated with the appropriate primary antibodies. The immunoreactive bands were detected using an enhanced chemiluminescence kit with Hyper-film (Amersham). Quantitative data normalized with internal control were obtained by using the computing densitometer and Multi Gauge v. 3.0 software (FujiFilm Life Science, Tokyo, Japan).

Semiquantitative Reverse Transcription-PCR (RT-PCR) Analysis. Total RNA was isolated from cells using TRIzol reagent (Invitrogen)

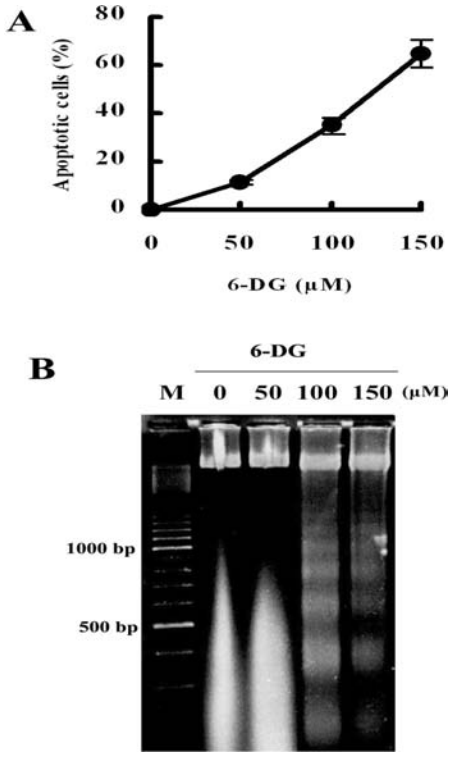

Figure 2. Effect of 6-DG on the induction of apoptosis in Hep G2 cells. (**A**) Flow cytometric analysis of apoptotic cells. Hep G2 cells were treated with 6-DG (50, 100, and 150 μM) for 24 h, and their DNA content was analyzed using flow cytometer after propidium iodide staining. The apoptotic fraction (sub-G_1) was indicated. Data are expressed as mean $\pm$ SD from three independent experiments. (**B**) DNA fragmentation (DNA ladder) in 6-DG-treated Hep G2 cells. Cells were treated with indicated concentrations of 6-DG for 24 h. Fragmented DNA was extracted and analyzed by agarose gel electrophoresis. M, size marker DNA. Data are representative of three independent experiments.

as instructed by the manufacturer. Two micrograms of RNA from each sample was used as a template for cDNA synthesis with a RNA PCR kit (TaKaRa, Japan). cDNA was amplified using the sense primer 5′-GACCTAGCTCCCCAGCAGAGAG-3′ and the antisense primer 5′-CGGCTGCAACTGTGACTCCTAT-3′ (corresponding to 403 and 490 bp regions of DR5). For glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the sense primer 5′-ACCACAGTCCATGCCATCAC-3′ and the antisense primer 5′-TCCACCACCCTGTTGCTGTA-3′ were used (corresponding to a 452 bp region of GAPDH). PCR was performed for 30 cycles. The conditions were as follows: (a) 50 s of denaturation at 94 °C, (b) 50 s of annealing at 58 °C for DR5 and GAPDH, and (c) 55 s of extension at 72 °C. This was followed by an additional extension step at 72 °C for 10 min. DNA products were electrophoresed on a 1.8% agarose gel at 100 V for 40 min and visualized with ethidium bromide staining under UV illumination. Quantitative data normalized with GAPDH internal control were obtained by using the computing densitometer and Multi Gauge v. 3.0 software (FujiFilm Life Science, Tokyo, Japan).

Statistics. All assays were carried out in triplicate. Data were analyzed to determine statistical significance of difference between the control and test group by Student's t test. A p value of < 0.05 was considered to be significant.

RESULTS

6-DG Induced Apoptosis in Hep G2 Cells. To investigate the apoptotic effect induced by 6-DG, Hep G2 cells were exposed to various concentrations of 6-DG for 24 h, and the extent of apoptotic cell death was assessed by the determination of sub-G_1 cell population in cells stained with propidium iodide. As shown in **Figure 2A**, 6-DG induced dose-dependent apoptotic cell

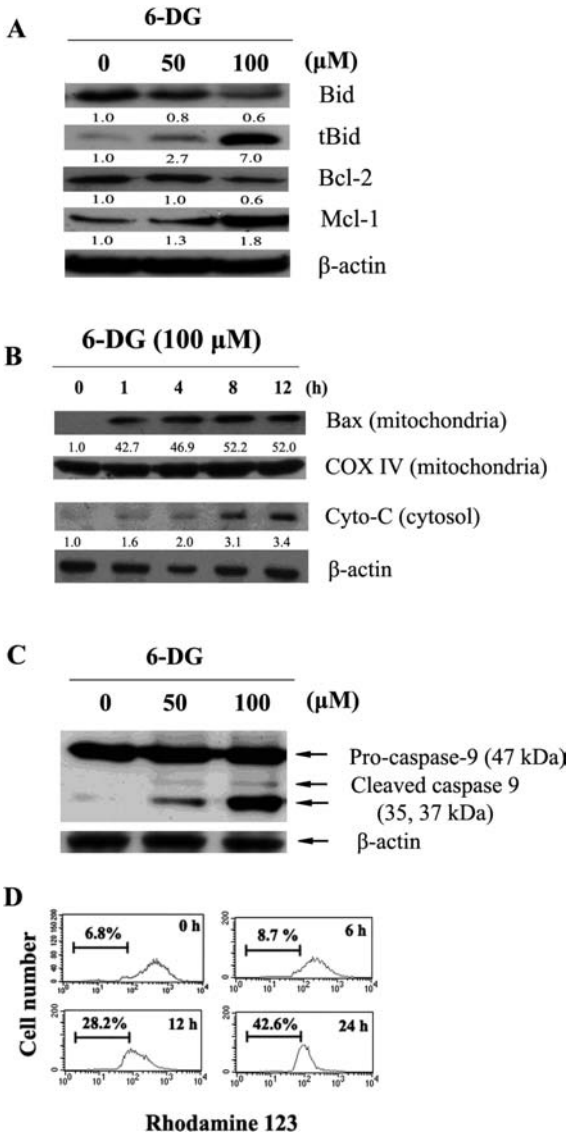

Figure 3. 6-DG-induced mitochondrial apoptotic pathway. (**A**) Effect of 6-DG on Bcl-2 family proteins. The levels of Bcl-2 family proteins (Bid, Bcl-2, and Mcl-1) were measured by Western blotting in Hep G2 cells treated with indicated concentrations of 6-DG for 24 h. Results are representative of three independent experiments. (**B**) Effect of 6-DG on the Bax and cytochrome c translocation. Cells were treated with 6-DG (100 μM) and harvested at indicated times. The mitochondrial and cytosolic proteins were prepared and subjected to Western blotting using antibodies against Bax, cytochrome c, COX IV, and β-actin. COX IV and β-actin were included as protein loading control for mitochondrial and cytosolic proteins, respectively. (**C**) Activation of caspase-9. Cleaved enzyme products of caspase-9 were measured by Western blotting in Hep G2 cells treated with indicated concentrations of 6-DG for 24 h. Results are representative of three independent experiments. (**D**) Effect of 6-DG on loss of MMP. Cells were treated with 6-DG (100 μM) and harvested at indicated times. The MMP was analyzed using flow cytometer after rhodamine 123 staining. Results are representative of three independent experiments.

death in Hep G2 cells. To further demonstrate that the demise of these 6-DG-treated cells was apoptotic in nature, we also performed DNA fragmentation studies. As shown in **Figure 2B**, a typical DNA ladder was found in Hep G2 cells after treatment with 6-DG.

6-DG Induced Mitochondrial Apoptotic Pathway. To determine whether the mitochondrial apoptotic signaling pathway was

involved in 6-DG-induced apoptosis, expression patterns of pro-apoptotic and anti-apoptotic Bcl-2 family proteins were investigated. As shown in **Figure 3A**, 6-DG exposure induced a significant reduction in expression of the BH3 domain-only protein Bid and a concomitant increase in expression of cleaved Bid (also known as truncated Bid (t-Bid), generated from the cleavage of inactive cytosolic Bid by caspase-8). In addition, 6-DG exposure also selectively decreased Bcl-2, but increased Mcl-1, protein levels (**Figure 3A**). This implied that Mcl-1 anti-apoptotic protein did not play a major role in 6-DG-induced apoptosis in Hep G2 cells. Bid-induced conformational change of Bax and its translocation to mitochondria are believed to play a key role in mitochondria-mediated apoptosis (*24*). To investigate whether the up-regulated expression of t-Bid could induce the mitochondrial apoptotic pathway in 6-DG-treated Hep G2 cells, subcellular localization of Bax and cytochrome *c* proteins, mitochondrial membrane potential (MMP), and the level of casapse-9 protein as well as its cleaved fragments were determined. Subcellular fractionation analysis showed that the increased levels of mitochondrial Bax and cytosolic cytochrome *c* were observed 1 h after the cells were exposed to 100 μM 6-DG (**Figure 3B**). These results implied that 6-DG induced Bax translocation to mitochondria and subsequently altered membrane permeability, rendering mitochondrial cytochrome *c* to be released to the cytosol. Next, we examined 6-DG-induced cleavage of caspase-9. Western blotting analysis revealed a concentration-dependent increment of caspase-9 cleavage p37 and p35 products in cells exposed to 6-DG (**Figure 3C**). The MMP was determined in Hep G2 cells after 6-DG treatment using the potential-sensitive dye rhodamine 123. As shown in **Figure 3D**, when Hep G2 cells were treated with 100 μM 6-DG for indicated time periods, the population that lost MMP was 28.2% at 12 h and 42.6% at 24 h. These results implied that mitochondrial apoptotic pathway was triggered in 6-DG-treated Hep G2 cells.

Fas-Mediated Death Receptor Pathway Was Activated in 6-DG-Treated Hep G2 Cells. To determine whether caspase-8 was involved in the increment of t-Bid in 6-DG-treated Hep G2 cells, caspase-8 activation was first examined by Western blotting using a monoclonal antibody that specially recognizes the active form of caspase-8. As shown in **Figure 4A**, concentration-dependent increments of caspase-8 cleavage p43 and p41 products were observed in cells exposed to 6-DG. Caspase-8 is activated by a death receptor-mediated pathway (*25*). To elucidate whether the Fas-mediated death receptor pathway was involved in caspase-8 activation, the levels of Fas and Fas ligand (Fas-L) were determined. As shown **Figure 4A**, a concentration-dependent increment of Fas, but not of Fas-L, was observed. In addition, we also examined the effect of Fas-L neutralizing antibody (NOK-1), which can disrupt Fas/Fas-L interaction, on 6-DG-induced formation of cleaved caspase-8. As shown in **Figure 4B**, the level of cleaved caspase-8 induced by 6-DG was reversed by Fas-L neutralizing antibody. Caspase-8 has been found to activate caspase-3 directly or indirectly (*25*). Hence, the effect of Fas-L neutralizing antibody (NOK-1) on 6-DG-induced formation of cleaved caspase-3 was examined. As shown in **Figure 4B**, Fas-L neutralizing antibody (NOK-1) could down-regulate the expression of cleaved caspase-3. These data suggested that Fas-mediated signaling pathway was activated in 6-DG-treated Hep G2 cells.

P53 Was Involved in 6-DG-Induced DR5 Expression and Enhancement of TRAIL-Induced Apoptosis. Although the Fas/Fas-L signaling pathway was activated in 6-DG-treated Hep G2 cells, the level of DR5 protein was also examined. As shown in **Figure 5A**, 6-DG up-regulated DR5 expression in a concentration-dependent manner, and the highest level of DR5 expression

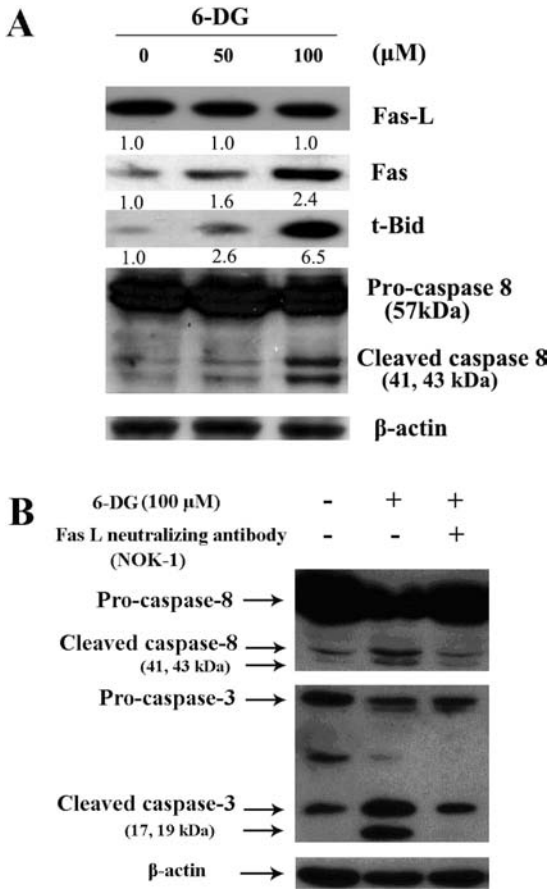

Figure 4. 6-DG-induced death receptor apoptotic pathway. (**A**) Levels of Fas-L, Fas, t-Bid, and cleaved enzyme products of caspase-8 measured by Western blotting in Hep G2 cells treated with indicated concentrations of 6-DG for 24 h. β-actin was included as a protein loading control. (**B**) Death receptor blocking experiment. Hep G2 cells were preincubated with Fas-L neutralizing antibody (NOK-1, 1 μg/mL) for 1 h before the addition of 100 μM 6-DG. Cleaved enzyme products of caspase-3 and caspase-8 were measured by Western blotting. β-actin was included as a protein loading control. Results are representative of three independent experiments.

was observed at a concentration of 150 μM; however, 6-DG at a concentration of 50 μM was sufficient to induce DR5 expression relative to untreated cells (**Figure 5A**). Therefore, 6-DG at a concentration of 50 μM was used to evaluate whether 6-DG-induced DR5 expression could enhance exogenous TRAIL-induced apoptosis. HepG2 cells were treated with 6-DG alone (50 or 100 μM), TRAIL alone (40 ng/mL), or a combination of 6-DG and TRAIL. Apoptotic death was assessed by the determination of sub-G_1 cell population in cells stained with propidium iodide. As illustrated in **Figure 5B**, combined treatment with 6-DG and TRAIL resulted in a markedly enhanced accumulation of sub-G_1. To determine whether 6-DG-induced DR5 up-regulation was controlled at the transcriptional level, we detected DR5 mRNA levels in cells exposed to different concentrations of 6-DG using RT-PCR. As shown in **Figure 5C**, 6-DG increased DR5 mRNA levels in a dose-dependent manner.

Recent studies have shown that the expression of DR5 is regulated by p53 (*15*−*17*). Post-translational modification of p53, such as phosphorylation at the Ser-15 residue, is closely related to its transcriptional activation (*11*). To elucidate the role of p53 in 6-DG-induced DR5 expression, Western blotting analysis was used to detect nuclear phosphorylated p53 (Ser-15) expression. As shown in **Figure 5D**, dose-dependent nuclear p53

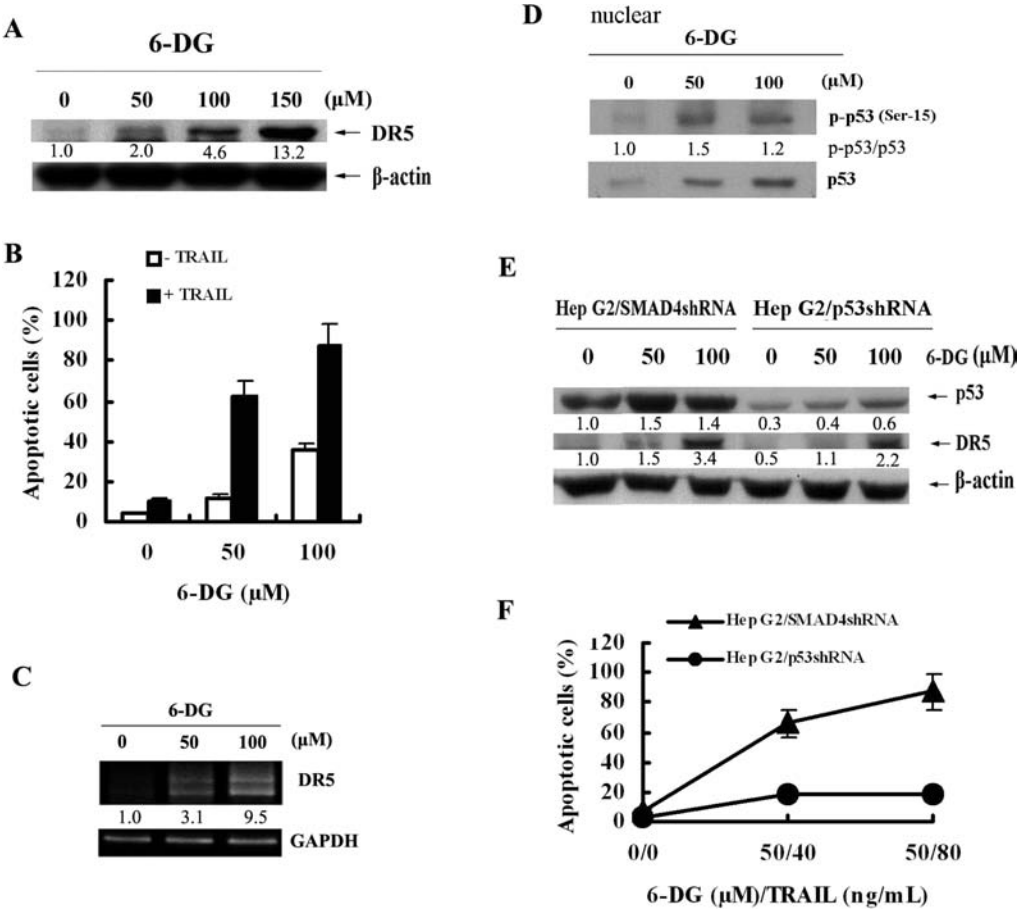

Figure 5. Involvement of p53 in 6-DG-induced DR5 expression and enhancement of TRAIL-induced apoptosis. (**A**) Hep G2 cells were treated with various concentrations of 6-DG (50, 100, and 150 μM) or DMSO (0.1%) alone for 24 h. Whole cell lysates were prepared and subjected to Western blotting using antibody against DR5. (**B**) Cells were treated with 6-DG alone (50 or 100 μM) or combined with TRAIL (40 ng/mL) for 24 h. Apoptotic death was assessed by the determination of sub-G_1 cell population as described under Materials and Methods. Data are expressed as mean ± SD from three independent experiments. (**C**) Hep G2 cells were exposed to various concentrations of 6-DG (50 and 100 μM) or DMSO (0.1%) alone for 24 h. Cellular total RNA was isolated, and RT-PCR analysis was performed as described under Materials and Methods. Results are representative of three independent experiments. Fold induction of DR5 mRNA normalized with GAPDH was quantified using Multi Gauge v. 3.0 software. (**D**) Cells were treated with various concentrations of 6-DG (50 and 100 μM) or DMSO (0.1%) alone for 24 h. Nuclear lysates were prepared and subjected to Western blotting using antibody against p53, phospho-p53 (Ser-15). Results are representative of three independent experiments. (**E**) Hep G2/SMAD4shRNA and Hep G2/p53shRNA cells were separately treated with various concentrations of 6-DG (50 and 100 μM) for 24 h. P53 and DR5 protein levels were determined by Western blotting. (**F**) Hep G2/SMAD4shRNA and Hep G2/p53shRNA cells were separately treated with a combined regimen of 6-DG (50 μM) and TRAIL (40 or 80 ng/mL) for 24 h. Apoptotic death was assessed by the determination of sub-G_1 cell population as described under Materials and Methods. Data are expressed as mean ± SD from three independent experiments.

phosphorylation at the Ser-15 residue was observed after 6-DG stimulation.

To further confirm the role of p53 in regulating DR5 expression, p53-knockdown Hep G2 cells (Hep G2/p53shRNA) and nonsilencing control cells (Hep G2/SMAD4shRNA) were treated with 6-DG. As shown in **Figure 5E**, 6-DG induced p53 and DR5 expression in Hep G2/SMAD4shRNA cells; however, little induction of p53 and down-regulated expression of DR5 were seen in Hep G2/p53shRNA cells.

Meanwhile, to confirm that p53-mediated DR5 expression indeed played an important role in 6-DG-enhanced TRAIL-induced apoptosis in Hep G2 cells, Hep G2/p53shRNA and Hep G2/SMAD4shRNA cells were separately treated with a combined regimen of 6-DG and TRAIL. As illustrated in **Figure 5F**, combined treatment with 6-DG and TRAIL resulted in a markedly enhanced accumulation of sub-G_1 in Hep G2/SMAD4shRNA cells, but an only slightly enhanced accumulation in Hep G2/p53shRNA cells. These data clearly indicate that 6-DG activated p53 signaling and induced downstream DR5 expression, thus sensitizing Hep G2 cells to TRAIL-induced apoptosis.

ROS Generation Was Involved in 6-DG-Induced DR5 Expression and Sensitization to TRAIL-Induced Apoptosis. To determine whether 6-DG-induced DR5 expression was associated with ROS-mediated oxidative stress, we first measured the intracellular ROS production by DCF fluorescence assay. As indicated in **Figure 6A**, when cells were exposed to 6-DG (100 μM), the level of intracellular ROS was drastically increased at 1 h and sustained for 12 h. Next, we examined the role of ROS induced by 6-DG in the up-regulation of DR5 and sensitization to TRAIL-induced apoptosis. As shown in **Figure 6B**, pretreatment with *N*-acetyl-L-cysteine (NAC) attenuated 6-DG-induced DR5 up-regulation and inhibited sub G_1 accumulation induced by a combined treatment of 6-DG and TRAIL from 60.5 to 8.3% (**Figure 6C**). Collectively, these results indicated that ROS generation induced by 6-DG was a prerequisite for the up-regulation of DR5 and sensitization to TRAIL-induced apoptosis.

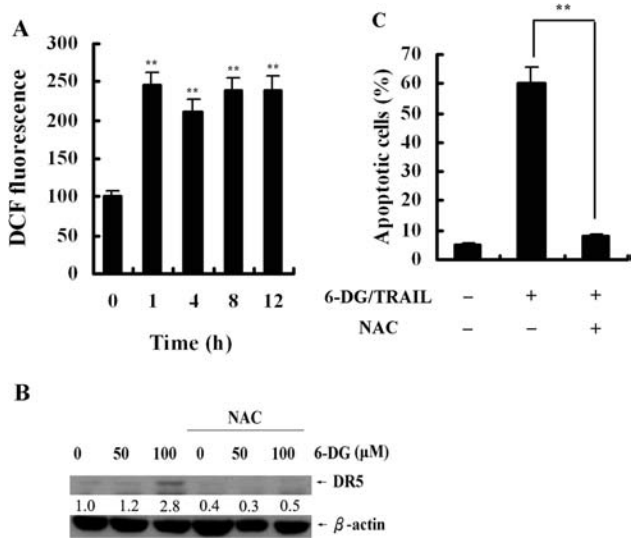

Figure 6. ROS generation was critical for 6-DG-induced DR5 expression and sensitization to TRAIL-induced apoptosis. (**A**) Cells were treated with 6-DG (100 μM) for indicated time periods and then harvested. Intracellular ROS content was evaluated by measuring oxidized DCF fluorescence using a flow cytometer. DCF fluorescence is presented as mean ± SD of three independent experiments. ** denotes the data that were significantly different from the control at $p < 0.01$. (**B**) Cells were pretreated with 10 mM NAC for 1 h followed by 6-DG (50 and 100 μM) treatment for 24 h. The levels of DR5 protein were determined by Western blotting. Results are representative of three independent experiments. (**C**) Cells were pretreated with 10 mM NAC for 1 h followed by the treatment with a combined regimen of 6-DG (50 μM) and TRAIL (40 ng/mL) for 24 h. The sub-G_1 fraction was determined as described under Materials and Methods. Data are expressed as mean ± SD from three independent experiments. ** denotes the data that were significantly different between the two groups at $p < 0.01$.

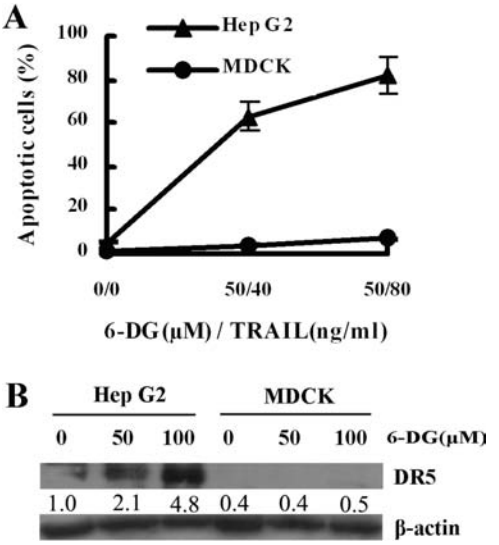

Figure 7. DR5 expression and sensitization to TRAIL-induced apoptosis were not observed in MDCK cells treated with 6-DG. (**A**) Hep G2 cells and MDCK cells were separately treated with a combined regimen of 6-DG (50 μM) and TRAIL (40 or 80 ng/mL) for 24 h. The sub-G_1 fraction was determined as described under Materials and Methods. Data are expressed as mean ± SD from three independent experiments. (**B**) Hep G2 cells and MDCK cells were separately treated with various concentrations of 6-DG (50 and 100 μM) for 24 h. The levels of DR5 protein were determined by Western blotting. Results are representative of three independent experiments.

6-DG Did Not Induce DR5 Expression and Failed To Enhance TRAIL-Induced Apoptosis in MDCK Cells. We showed that 6-DG could up-regulate DR5 expression and enhance TRAIL-induced apoptosis in Hep G2 cells. Next, we examined the effect of a combined treatment on normal cells. MDCK cells were selected as a model. Our results showed that the combination of 6-DG and TRAIL did not induce apoptosis in normal MDCK cells, although it markedly induced apoptosis in Hep G2 cells (**Figure 7A**). We then examined whether the resistance of MDCK cells to a combined treatment was attributed to the differential expression of DR5. As shown in **Figure 7B**, DR5 was not up-regulated by 6-DG in MDCK cells, whereas it was up-regulated in Hep G2 cells.

DISCUSSION

In this study, we have demonstrated for the first time that 6-DG, a constituent isolated from the rhizomes of *Z. officinale*, was capable of inducing apoptosis in human hepatoblastoma Hep G2 cells, a well-differentiated and characteristic strong drug-resistant subline (*26*). Experiments designed to gain insight into the molecular mechanisms underlying the 6-DG-induced apoptosis in Hep G2 cells unveiled that 6-DG could simultaneously induce both mitochondria- and death receptor-mediated apoptotic pathways in Hep G2 cells.

Death receptor-mediated apoptotic pathways have been considered as important signals for apoptotic cell death (*27*). In this study, we showed that 6-DG treatment resulted in Fas receptor up-regulation, caspase-8 activation, and t-Bid formation (**Figure 4A**). Moreover, by using Fas-L neutralizing antibody (NOK-1), we found that the formation of cleaved caspase-8 and caspase-3 could be inhibited by Fas-L neutralizing antibody (NOK-1) (**Figure 4B**). These data suggested that the Fas/Fas-L pathway was involved in 6-DG-induced apoptosis in Hep G2 cells.

Truncated Bid, the cleavage product of Bid by caspase-8 following engagement of Fas death receptor, has been shown to induce oligomerization of Bax and lead to cytochrome *c* release (*28*, *29*). In this study, we showed that 1 h after 6-DG treatment, Bax, a pro-apoptotic protein, redistributed to the outer mitochondrial membrane, which was coincident with the timing of cytochrome *c* leakage to the cytosol, and subsequently activated caspase-9 (**Figure 3B**,**C**). In addition, a collapse of MMP was also found 12 h after 6-DG stimulation (**Figure 3D**). These data suggested that t-Bid, generated from the Fas/Fas-L pathway, could activate the mitochondrial apoptotic pathway in 6-DG-treated Hep G2 cells.

An untagged recombinant human TRAIL (amino acids 114−281) has been proven to be nontoxic to normal human hepatocytes and is currently being developed as a clinical agent for the treatment of some liver diseases (*30*). However, recent studies have shown that some human hepatocellular carcinoma (HCC) cells were resistant to TRAIL-induced apoptosis (*11*, *12*, *31*, *32*). Thus, TRAIL sensitizers that are capable of overcoming TRAIL resistance are currently being investigated. DR5, a member of the TNF receptor superfamily, was known to ligate with TRAIL and triggered death receptor-mediated apoptotic pathway. Therefore, an agent that can up-regulate DR5 expression may be a promising candidate for sensitizing HCC cells to TRAIL-induced apoptosis. In the present study, we showed that 6-DG could sensitize Hep G2 cells to TRAIL-induced apoptosis through the up-regulated expression of DR5.

Phosphorylation at the Ser-15 residue of p53 is critical for p53-dependent transactivation, leading to apoptosis induced by DNA

damage agents (*14*). Recently, DNA damage agents were also reported to induce DR5 expression in a p53-dependent manner (*15*−*17*). To delineate the role of p53 in the regulation of DR5 expression, the levels of these two proteins were first determined in 6-DG-treated Hep G2 cells. Our results showed that 6-DG could induce nuclear translocation of p53 and its phosphorylation at Ser-15 residue, which correlated with the extent of DR5 expression, implying that p53 might be involved in the up-regulation of DR5. Abrogation of p53 expression by p53 small interfering RNA could significantly inhibit DR5 expression (**Figure 5E**), indicating the crucial role of p53 in the regulation of DR5 expression. More importantly, the fact that 6-DG in combination with TRAIL could cause severe apoptosis in HepG2/SMAD4shRNA cells in comparison to HepG2/p53shRNA cells (**Figure 5F**) further strengthened that p53 played a pivotal role in 6-DG-induced DR5 up-regulation and sensitization to TRAIL-induced apoptosis. The slight increase in DR5 levels in 6-DG-treated p53-knockdown HepG2/p53shRNA cells could be due to the residual p53.

Several chemopreventive agents and proteosome inhibitors have been found to induce ROS-dependent DR5 expression and enhancement of TRAIL-induced apoptosis (*4*−*6*). In the present study, we found that pretreatment with ROS scavenger NAC could significantly decrease DR5 expression (**Figure 6B**). Moreover, our results also showed that NAC could reverse 6-DG-enhanced TRAIL-induced apoptosis. Taken together, these results suggested that ROS production was involved in DR5 expression and thus enhanced the TRAIL-induced apoptosis in 6-DG-treated Hep G2 cells.

Anticancer drugs, which are successfully applied in the field of cancer therapy, are mainly relied on for their abilities to selectively eradicate malignant cells, but not normal cells. Here we found that 6-DG did not induce DR5 expression and enhanced sensitization to TRAIL-induced apoptosis in normal MDCK cells, albeit that hepatoblastoma Hep G2 cells underwent severe apoptosis when a combined regimen was employed. The tumor selectivity effect of a combined regimen of 6-DG and TRAIL may provide a new alternative strategy for tumor eradication. However, prior to the clinical utilization, further in vivo appraisal of potential toxicity of this combined regimen is urgently needed.

MDCK cells derived from normal canine kidneys have been widely used to study drug-induced renal toxicities (*33*). To evaluate the renal toxicity and tumor selectivity of the combined regimen of 6-DG and TRAIL, MDCK cells were selected as a comparing normal cell line. As shown in **Figure 7A**, the combined regimen of 6-DG and TRAIL showed no renal toxicity, but did cause selective eradication of hepatoblastoma Hep G2 cells.

In conclusion, we have shown that 6-DG up-regulated DR5 expression through a p53-dependent pathway, and this strategy could synergistically enhance TRAIL-induced apoptosis. Therefore, a combined regimen of 6-DG and TRAIL may provide a promising strategy for safe treatment of tumor cells carrying wild type p53.

ABBREVIATIONS USED

TRAIL, TNF-related apoptosis-inducing ligand; DR5, death receptor 5; 6-DG, 6-dehydrogingerdione; ROS, reactive oxygen species; NAC, *N*-acetyl-L-cysteine; TRAIL, tumor necrosis factor-related apoptosis-inducing factor; DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate.

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