Dioscin sensitizes cells to TRAIL-induced apoptosis through downregulation of c-FLIP and Bcl-2

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Abstract. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has received attention as a potential anti-cancer drug, because it induces apoptosis in a wide variety of cancer cells but not in most normal human cell types. Here, we showed that co-treatment with subtoxic doses of dioscin and TRAIL-induced apoptosis in Caki human renal cancer cells. Treatment of Caki cells with dioscin down-regulated c-FLIP$_L$ and Bcl-2 proteins in a dose-dependent manner. Dioscin-induced decrease in c-FLIP$_L$ protein levels may be caused by the increased protein instability. We also found that dioscin induced downregulation of Bcl-2 at the transcriptional level. Pretreatment with NAC slightly inhibited the expression levels of c-FLIP$_L$ downregulated by the treatment of dioscin, suggesting that dioscin is partially dependent on the generation of ROS for downregulation of c-FLIP$_L$. Taken together, the present study demonstrates that dioscin enhances TRAIL-induced apoptosis in human renal cancer cells by downregulation of c-FLIP$_L$ and Bcl-2.

Introduction

TRAIL (tumor necrosis factor (TNF)-related apoptosis-inducing ligand) belongs to the TNF superfamily, which can induce apoptosis in a wide variety tumor cells but not normal cells (1). Because of its ability, TRAIL is showing promise as a cancer therapeutic agent. TRAIL induces apoptosis through interacting with death receptor 4 (DR4; TRAIL-R1) and death receptor 5 (DR5; TRAIL-R2) leading to the formation of the death-inducing signal complex (DISC) with binding of caspase-8, leading to apoptosis (extrinsic or death receptor pathway) (1,2). In addition, TRAIL induces apoptosis via the disruption of the mitochondria membrane permeability, release of cytochrome c into the cytoplasm and activation of caspase-9 (intrinsic or mitochondria pathway) (3). Despite the beneficial effect of TRAIL to selectively kill tumor cells, many cancer cells appear to show resistance to TRAIL (2). The mechanism of TRAIL resistance is not clearly, but several studies has been reported that TRAIL resistance is intimately associated with overexpression of anti-apoptosis including FADD-like apoptosis regulator (c-FLIP), anti-apoptotic Bcl-2 family proteins (e.g., Bcl-2 and Bcl-xL) and inhibitor of apoptosis proteins (IAPs) (2). However, single treatment with TRAIL may not be sufficient for the treatment of various malignant tumor cells, TRAIL-resistant cancer cells can be sensitized by TRAIL sensitizer such as chemotherapeutic drugs and biochemical inhibitors that suppress the expression of anti-apoptosis-associated proteins including Bcl-2, c-FLIP or XIAP, indicating that combination therapy may be a possibility. Therefore, understanding the molecular mechanisms of TRAIL resistance and ways to sensitize these cells to undergo apoptosis by TRAIL are important issues for effective cancer therapy.

Dioscin, a plant glucoside saponin extracted from the roots of *Polygonatum zanlanscianense*, has anti-inflammatory, lipid-lowering, anticancer and hepatoprotective effects (4-7). Several mechanisms have been proposed for the anti-cancer activity of dioscin, including induction of apoptosis and arrest of cell cycle (8,9). Dioscin-induced apoptosis were mediated by activation of caspase-9 and -3, together with downregulation of anti-apoptotic Bcl-2 protein (8,10) or by the elevated oxidative stress mediated by downregulation of peroxiredoxins as well as through mitochondria dysfunction (5,11).

The aim of this study is to evaluate dioscin as a sensitizer of TRAIL and to understand the mechanism of the synergy between dioscin and TRAIL against human renal cancer cells.

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Dioscin treatment rendered human renal cancer cells more sensitive to TRAIL. These results suggest that this combined treatment with dioscin and TRAIL may provide a safe and effective therapeutic strategy against malignant cancer that are resistant to various conventional treatments. Furthermore, we provide novel evidence that the prominent sensitizing effect of dioscin on TRAIL-induced apoptosis is due to ROS generation which causes downregulation of c-FLIP.

Materials and methods

Cells and materials. The Caki cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The culture medium used throughout these experiments was Dulbecco's modified Eagle's medium (DMEM), containing 10% fetal calf serum (FCS), 20 mM HEPES buffer and 100 μg/ml of gentamycin. Anti-Bcl-2, anti-PARP, anti-pro-caspase-3, anti-Mcl-1, and anti-actin antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Anti-c-FLIP antibody was purchased from Alexis Corp. (San Diego, CA, USA). Dioscin was isolated from *Polygonatum zanlanscianse* PAMP) and were directly added to cell cultures at the indicated concentrations. N-acetyl-L-cysteine (NAC) and pan-caspase inhibitor (Z-VAD-FMK) were purchased from Calbiochem (San Diego, CA, USA).

Purification and identification of dioscin. The root of *Dioscorea nipponica* Makino was obtained from the Uiseong Medicinal Farm (Uiseong, Korea). Three kilograms of the roots were extracted three times with 5 liters of methanol each time. After filtration, the extract was evaporated in vacuo to give 115 g of dry sample. The following procedures of purification of dioscin based on silica-gel chromatography were the same as previously reported (12). The purified compound was identified as dioscin by analyses of IR spectroscopy (Perkin-Elmer, Shelton, CT, USA) and 1H- and 13C-NMR spectroscopy (Bruker AMX 300, Rheinsten, Germany).

HPLC analysis of dioscin. The purity of dioscin was confirmed by HPLC analysis as was previously reported (12). Dioscin and its derivatives, such as prosapogenin A and prosapogenin C were determined by HPLC system comprising an SCL-10A system controller, LC-10AD pump and SPD-10A UV detector (Shimadzu, Japan). The analytical column was a Mightysil RP-C18 GP-250 (Kanto Chemical Co., USA). The mobile phase for HPLC consisted of 75% acetonitrile (v/v) with a flow rate of 0.7 ml/min. The column temperature was maintained at 30˚C. A 10 μl of the sample dissolved in methanol (1 mg/ml) was injected into the HPLC system, and the UV absorption at 215 nm was recorded. The retension time of dioscin was 3.25 min and the purity of dioscin was identified as above 98.5%.

Western blotting. Cellular lysates were prepared by suspending $6x10^5$ cells in 100 μl of lysis buffer (137 mM NaCl, 15 mM EGTA, 0.1 mM sodium orthovanadate, 15 mM $MgCl_2$, 0.1% Triton X-100, 25 mM Mops, 100 μM phenylmethlsulfonyl fluoride, and 20 μM leupeptin, adjusted to pH 7.2). The cells were disrupted by sonication and extracted at 4˚C for 30 min. Lysates containing proteins were quantified using BCA protein assay kit (Pierce, Rockford, IL, USA). The proteins were electrotransferred to Immobilon-P membranes (Millipore Corp., Bedford, MA, USA). Detection of specific proteins was carried out with an ECL western blotting kit (Millipore) according to the manufacturer's instructions.

Cell count and flow cytometry analysis. Cell counts were performed using a hemocytometer. Approximately $1x10^6$ Caki cells were suspended in 100 μl of PBS, and 200 μl of 95% ethanol were added while vortexing. The cells were incubated at 4˚C for 1 h, washed with PBS, and resuspended in 250 μl of 1.12% sodium citrate buffer (pH 8.4) together with 12.5 μg of RNase. Incubation was continued at 37˚C for 30 min. The cellular DNA was then stained by applying 250 μl of propidium iodide (50 μg/ml) for 30 min at room temperature. The stained cells were analyzed by fluorescent activated cell sorting (FACS) on a FACScanto flow cytometer for relative DNA content based on red fluorescence.

RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR). Total cellular RNA was extracted from cells using the Easy-blue Total RNA Extraction kit (iNtRon, Sungnam, Korea). A cDNA was synthesized from 5 μg of total RNA using M-MLV reverse transcriptase (Promega, Madison, WI, USA). The cDNAs for c-FLIP, Bcl-2 and actin were amplified by PCR with specific primers. The sequence of the sense primer for c-FLIPL was 5'-CGG ACT ATA GAG TGC TGA TGG-3' and the antisense primers were 5'-GAT TAT CAG GCA GAT TCC TAG-3'. PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide.

Statistical analysis. Three or more separate experiments were performed. Statistical analysis was done by paired Student's t-test or ANOVA. A P-value <0.05 was considered to have pronounced difference between experimental and control groups.

Results

Dioscin treatment induces apoptosis in a dose-dependent manner in Caki cells. To investigate the effect of dioscin-induced apoptosis, human renal carcinoma Caki cells were treated with various concentrations of dioscin. Two established criteria were subsequently used to assess apoptosis in this study. Apoptosis was determined in Caki cells using flow cytometry analysis demonstrating hypo-diploid DNA. Fig. 1A shows treatment with dioscin in Caki cells resulted in a markedly increased accumulation of sub-G1 phase in a dose-dependent manner of dioscin. Because cells undergoing apoptosis executed the death program by activating caspases and cleavage of PARP, we analyzed expression levels of pro-caspase-3, and cleavage of PARP. As demonstrated in Fig. 1B, exposure to dioscin led to a reduction of the 32-kDa precursor, accompanied by a concomitant revealed cleavage of PARP. Next, we analyzed nuclear condensation, which is another hallmark of apoptosis. Combinatory treatment with dioscin plus TRAIL induced nuclear condensation in Caki cells. In contrast, nuclear condensation in Caki cells treated with TRAIL alone or dioscin alone was barely detected.

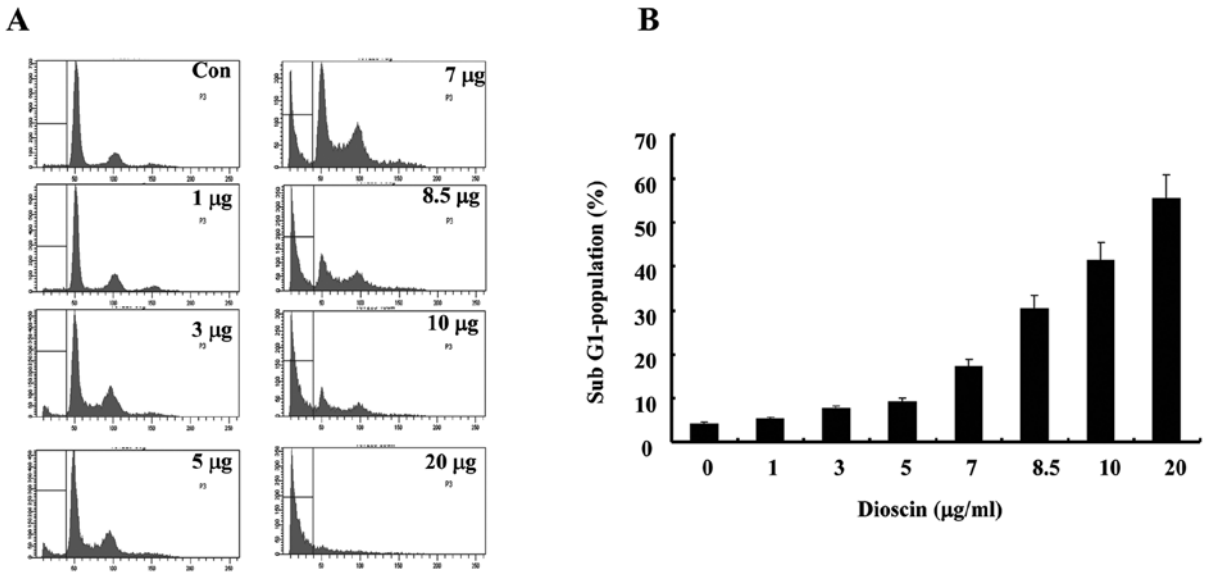

Figure 1. Dioscin treatment induces apoptosis in a dose-dependent manner in Caki cells. (A) Flow cytometric analysis of apoptotic cells (histogram). Caki cells were treated with the indicated concentrations of dioscin for 24 h. Apoptosis was analyzed as a sub-G1 fraction by FACS. (B) Flow cytometric analysis of apoptotic cells (Graph).

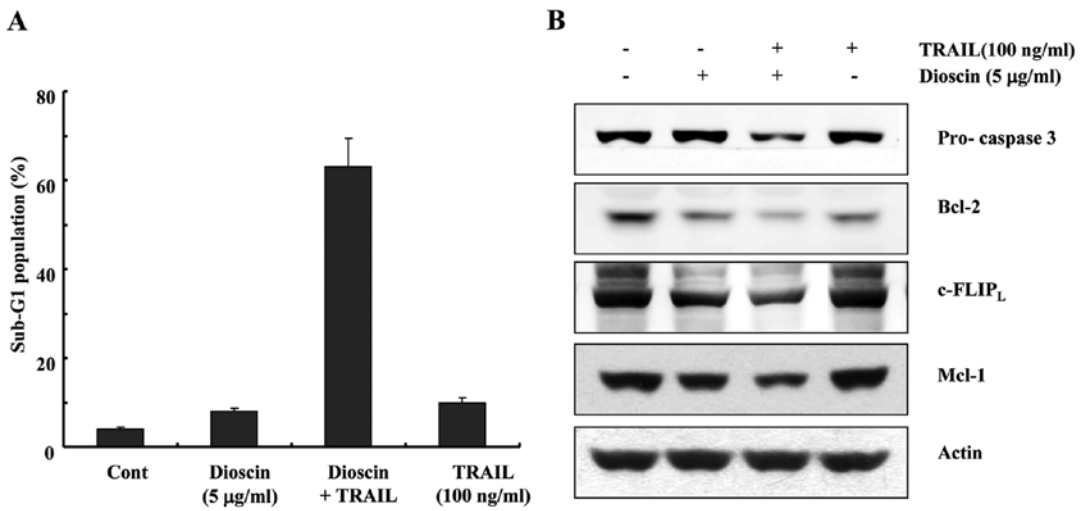

Figure 2. Dioscin sensitizes renal cancer cells to TRAIL-mediated apoptosis. (A) Caki cells were treated for 24 h with TRAIL (100 ng/ml) in either the absence or the presence of dioscin (5 μg/ml). After 24 h, apoptosis was analyzed as a sub-G1 fraction by FACS. (B) Activation of caspases in dioscin-sensitized TRAIL-induced apoptosis. Cells were treated with the indicated concentrations of dioscin and TRAIL. Equal amounts of cell lysates (40 μg) were subjected to electrophoresis and analyzed by western blot analysis for c-FLIP, Bcl-2, Mcl-1 pro-caspase-3, and actin for normalization.

Dioscin sensitizes renal cancer cells to TRAIL-mediated apoptosis. In an attempt to search for novel strategies to overcome TRAIL resistance in cancer cells, we investigated the effect of the combined treatment with dioscin and TRAIL in Caki cells. Co-treatment of Caki cells with dioscin and TRAIL resulted in a markedly increased accumulation of sub-G1 phase cells, compared with Caki cells treated with dioscin or TRAIL alone (Fig. 2A). In addition, combinatory treatment of Caki cells with dioscin and TRAIL strongly stimulated reduction of the protein levels of pro-caspases 3, Bcl-2, Mcl-1, and c-FLIP$_L$ (Fig. 2B).

Dioscin downregulates Bcl-2, Mcl-1 and c-FLIP protein expressions. To investigate the underlying mechanisms involved in dioscin enhanced TRAIL-induced apoptosis, we analyzed the changes in the expression levels of various apoptosis-regulating proteins. Bcl-2, Mcl-1 and c-FLIP$_L$ protein expressions were decreased by the indicated concentrations of dioscin-treated Caki cells in a dose-dependent manner. To further elucidate the mechanism responsible for the changes in amounts of proteins level, we determined the levels of Bcl-2, Mcl-1 and c-FLIP$_L$ mRNAs by RT-PCR. c-FLIP$_L$ and Mcl-1 mRNA levels remain constant through the dioscin treatment at different doses in Caki cells. We found that dioscin treatment of Caki cells dose-dependently decreased the mRNA levels of Bcl-2 from RT-PCR analysis, suggesting that dioscin modulates Bcl-2 expression at the transcriptional level and c-FLIP$_L$ and Mcl-1 at the post-transcriptional level (Fig. 3).

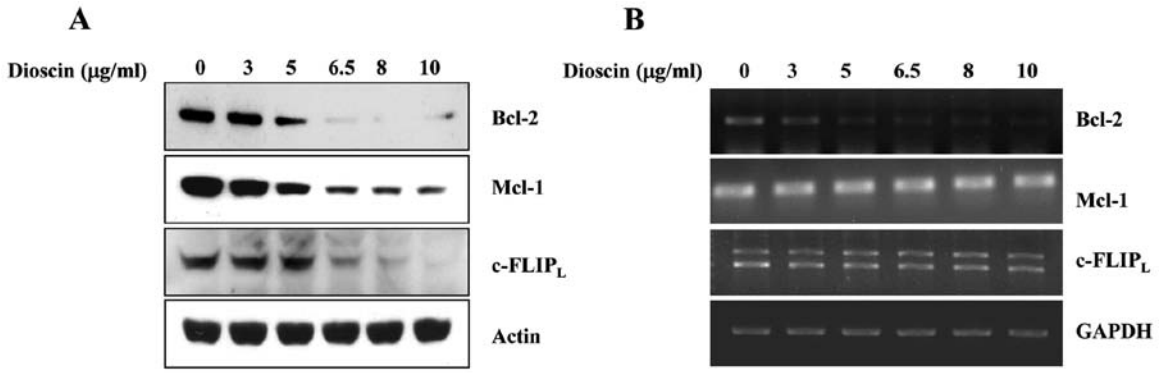

Figure 3. Dioscin modulates Bcl-2 expression at transcriptional level. (A) Caki cells were treated with the indicated doses for 24 h and harvested in lysis buffer and equal amounts of cell lysates (40 μg) were resolved by SDS-PAGE. Western blotting was performed using anti-cFLIP, -Bcl-2, or with anti-actin antibody as a control for the loading of protein level. (B) Caki cells were treated with the indicated concentrations of dioscin for 24 h. Total RNA was isolated and RT-PCR analysis was performed as described in Materials and methods. A representative study is shown; two additional experiments yielded similar results.

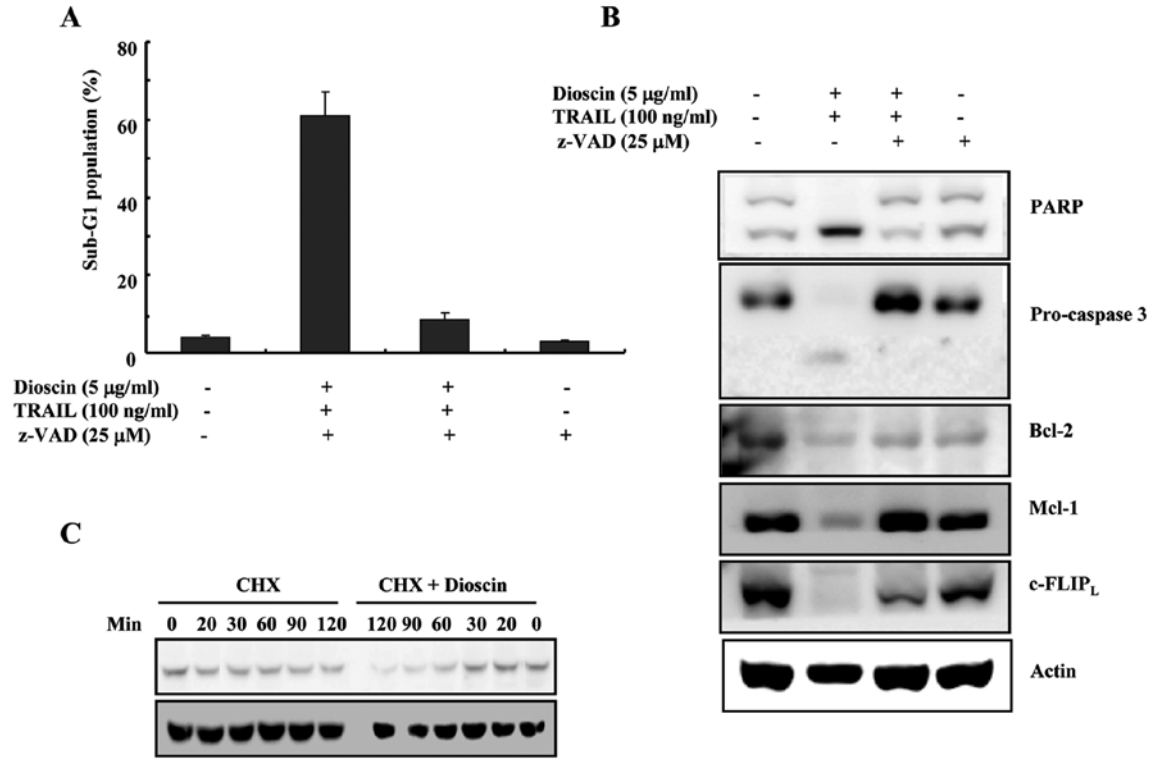

Figure 4. Combinatory treatment with dioscin plus TRAIL-induced apoptosis was mediated via caspase-dependent pathway. (A) Effect of z-VAD-fmk on apoptosis induced by dioscin plus TRAIL. Caki cells were incubated with 50 μM z-VAD-fmk or solvent for 1 h before treatment with dioscin (5 μg/ml) and/or TRAIL (100 ng/ml) for 24 h. DNA contents of treated cells were evaluated after propidium iodide staining and apoptosis was measured as a sub-G1 fraction by FACS. (B) Effect of z-VAD-fmk on caspase activation in dioscin plus TRAIL treated cells. Cells were treated with the indicated concentrations of dioscin and TRAIL. Equal amounts of cell lysates (40 μg) were subjected to electrophoresis and analyzed by western blot analysis for pro-caspase-3, PARP, c-FLIP, Mcl-1, Bcl-2, and actin to serve as control for the loading of protein level. A representative study is shown; two additional experiments yielded similar results. (C) Caki cells were treated with 5 μg/ml of dioscin in the presence or absence of CHX for the indicated times. Western blotting was performed using anti-c-FLIP antibody and anti-actin antibody served as control for the loading of protein level.

Dioscin plus TRAIL-induced apoptosis was mediated via caspase-dependent pathway. We next examined whether activation of caspase pathway plays a critical role in dioscin plus TRAIL-induced apoptosis. As shown in Fig. 4A, dioscin plus TRAIL-induced apoptosis was completely prevented by pre-treatment with a general and potent inhibitor of caspases, z-VAD-fmk, as determined by FACS analysis. These results suggest that the combined treatment with dioscin and TRAIL-induced apoptosis was mediated by caspase-dependent apoptosis pathways. We also found that z-VAD-fmk prevented all these caspase-related events such as cleavage of pro-caspase-3 and PARP (Fig. 4B). Pretreatment with z-VAD-fmk recovered Mcl-1 protein which were downregulated by combination treatment with dioscon plus TRAIL to basal level, but z-VAD-fmk partly blocked dioscin plus TRAIL-induced downregulation of c-FLIP$_L$ protein, indicating that the decreased c-FLIP$_L$ protein level was partly caused by caspase activation. These results suggested the possibilities that the

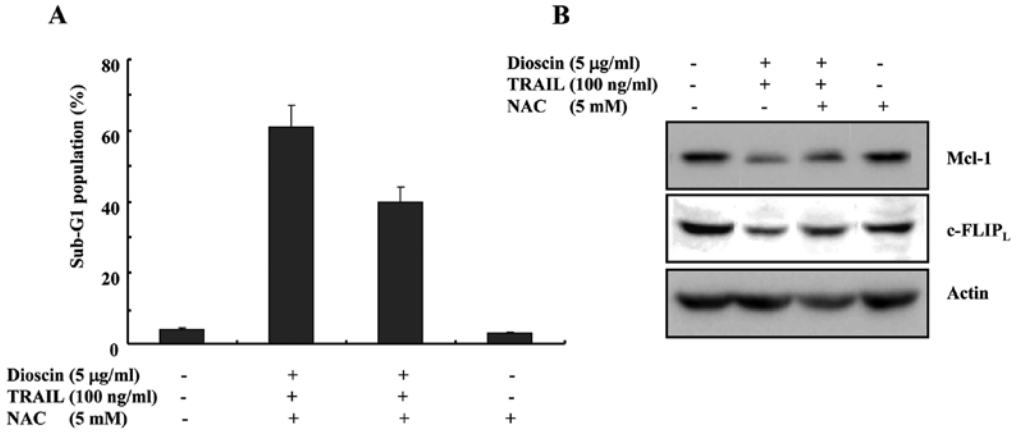

Figure 5. Dioscin-stimulated TRAIL-induced apoptosis appears to be partially dependent on the formation of ROS via downregulation of c-FLIP$_L$. (A) Effect of NAC on apoptosis induced by dioscin plus TRAIL. Caki cells were incubated with 5 mM NAC or solvent for 1 h before treatment with dioscin (5 μg/ml) and/or TRAIL (100 ng/ml) for 24 h. DNA contents of treated cells were evaluated after propidium iodide staining and apoptosis was measured as a sub-G1 fraction by FACS. (B) Pretreatment with NAC attenuated dioscin-induced downregulations of c-FLIP$_L$. Western blot analysis was performed as above.

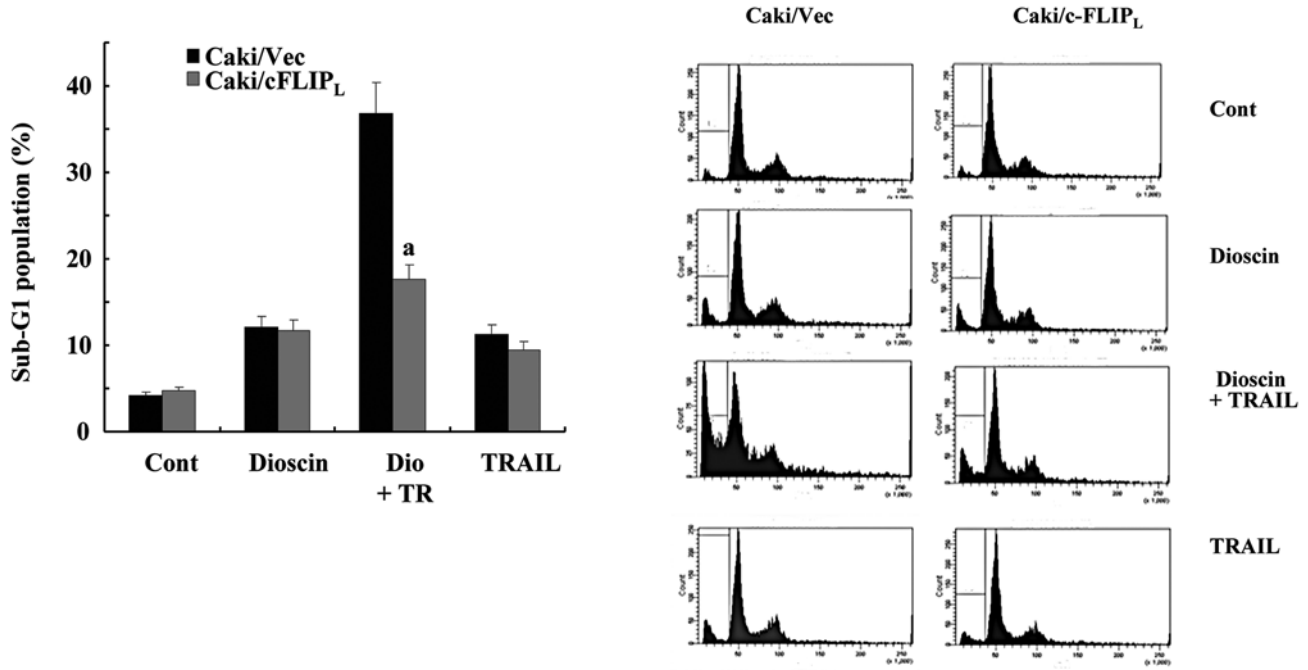

Figure 6. Downregulation of c-FLIP$_L$ contributes to dioscin-stimulated TRAIL-induced apoptosis. (A) Caki/Vector and Caki/c-FLIP$_L$ cells were treated for 12 h with dioscin alone (5 μg/ml), TRAIL alone (100 ng/ml), or combination of dioscin and TRAIL. Apoptosis was analyzed as a sub-G1 fraction by FACS. Data shown are means ± SD (n=3). [a]P<0.05 compared to dioscin plus TRAIL-treated pcDNA cells. The FACS data (histogram) are shown in the right panel. (B) Equal amounts of cell lysates (40 μg) were subjected to electrophoresis and analyzed by western blot analysis for PARP, procaspase-3, and c-FLIP$_L$.

decreased c-FLIP$_L$ protein was partly caused by caspase-independent pathways (Fig. 4B).

To further clarify the underlying mechanisms of the decreased c-FLIP$_L$ protein level in dioscin-treated cells, we performed c-FLIP$_L$ protein stability test. Caki cells were treated with cycloheximide (CHX) and dioscin for different doses. We found that the degradation of c-FLIP$_L$ protein was facilitated by dioscin treatment (Fig. 4C), implying that dioscin treatment caused reduction of c-FLIP protein stability.

Dioscin-stimulated TRAIL-induced apoptosis appears to be partially dependent on the formation of reactive oxygen species (ROS) via downregulation of c-FLIP$_L$ and Bcl-2. Numerous investigations have documented that ROS may play an important role during apoptosis induction (13,14). It has been reported that dioscin increases ROS production in various cancer cells (5,10). Therefore, we investigated whether ROS generation is directly associated with dioscin plus TRAIL-induced apoptosis. As shown in Fig. 5A, dioscin plus TRAIL-induced apoptosis was completely prevented by pretreatment with NAC, as determined by FACS analysis. As shown in Fig. 5B, pretreatment with NAC decreased the increased expression levels of c-FLIP$_L$ and Bcl-2 by dioscin treatment to basal levels, dioscon-induced downregulation of c-FLIPL protein was partly blocked by NAC treatment.

Downregulations of c-FLIP$_L$ contribute to dioscin-stimulated TRAIL-induced apoptosis. We examined whether dowregulation of c-FLIP$_L$ by dioscon is critical to stimulate TRAIL-induced apoptosis. Overexpression of c-FLIP$_L$ in Caki cells significantly attenuated dioscin-facilitated TRAIL-induced apoptosis, whereas co-treatment with dioscin plus TRAIL induced significant apoptosis in Caki/vector cells (Fig. 6). This result suggests that c-FLIP$_L$ downregulation also contributes to dioscin-facilitated TRAIL-induced apoptosis.

Discussion

In this study, we demonstrated for the first time that combination treatment with dioscin plus TRAIL on renal cancer cells synergistically induced apoptosis. Dioscin-mediated dowregulation of Bcl-2 is controlled at the transcriptional level in a dose-dependent manner. In contrast, dioscin-induced downregulation of c-FLIP$_L$ is caused by facilitating degradation of c-FLIP$_L$ protein. In addition, we also found that production of ROS by dioscin treatment seemed to partially take part in c-FLIP$_L$ downregulation.

Several reagents such as compound C, rosiglitazone, LBH589, and silibinin can induce downregulation of c-FLIP and subsequent sensitization to TRAIL-induced apoptosis in different cancer cells (15-18). It is generally recognized that c-FLIP$_L$ protein levels can be regulated by ubiquitin/proteasome mediated degradation (19,20) or by their transcriptional control through the NF-κB or c-Fos pathway (21,22). In this study, dioscin promotes ubiquitin/proteasome-mediated degradation of c-FLIP$_L$, leading to downregulation of c-FLIP, but not by transcriptional control. However, further work is needed for the mechanistic study to elucidate dioscin-induced activation of the proteasomal signaling pathway.

It has been suggested that cells can regulate proteasome function in response to increased ROS level both by altering the total number of proteasomes and by altering the subunit components of the ubiquitin-proteasome (23). Dioscin sensitizing HL-60 cells to apoptosis through a ROS-dependent mechanism is supported by direct measurement of ROS generation (5). Recently, several studies have shown that ROS downregulates c-FLIP levels and increases the sensitivity to apoptotic stimuli (24,25). Therefore, we investigated whether downregulations of c-FLIP$_L$ was actually mediated by ROS signaling pathway. In the presence of NAC, the decreased levels of c-FLIP$_L$ caused by dioscin were partly restored. Taken together, dioscin-stimulated TRAIL-induced apoptosis appears to be dependent on the formation of ROS for downregulations of c-FLIP$_L$.

Recently, it has been suggested that cytotoxicity of dioscin was mediated by activating death receptor through upregulation of Fas, FasL (Fas ligand), TNF-α, TNF receptor-1, and TNF receptor-associated factor 1 as well as by activating mitochondrial pathways through downregulation of Bcl-2 and in human gastric carcinoma cells (26). However, we found that the expression of Bcl-2 was downregulated by dioscin treatment at transcriptional level in our study, the expression level of TRAIL death receptor (DR5) was not altered by dioscin treatment.

In summary, we suggest that the use of dioscin is a potentially important therapeutic approach for enhancing sensitivity to TRAIL via downregulation of proteins related to the inhibition of the apoptotic processes such as Bcl-2 and c-FLIP.

Acknowledgements

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