Gambogic acid induces apoptosis and sensitizes TRAIL-mediated apoptosis through downregulation of cFLIP$_L$ in renal carcinoma Caki cells

JI HOON JANG*, JOO-YOUNG KIM*, EON-GI SUNG, EUN-AE KIM and TAE-JIN LEE

Department of Anatomy, College of Medicine, Yeungnam University, Nam-gu, Daegu 705-717, Republic of Korea

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Abstract. Gambogic acid (GA) is a natural compound derived from brownish gamboge resin that shows a range of bioactivity, such as antitumor and antimicrobial properties. Although, GA is already known to induce cell death in a variety of cancer cells, the molecular basis for GA-induced cell death in renal cancer cells is unclear. In this study, a treatment with GA induced cell death in human renal carcinoma Caki cells in a dose-dependent manner. Treatment of Caki cells with GA decreased the levels of antiapoptotic proteins, such as Bcl-2 and XIAP in a dose-dependent manner. In addition, GA decreased the expression of the cFLIP$_L$ protein, which was downregulated at the transcriptional level without any change in the levels of cFLIPs expression. z-VAD (pan-caspase inhibitor) partially blocked GA-mediated cell death. GA-induced apoptotic cell death in Caki cells is mediated partly by the AIF translocation from the mitochondria into the nucleus via a caspase-independent pathway. In contrast, N-acetylcysteine (NAC), a ROS scavenger, had no effect on GA-induced cell death. The restoration of cFLIP$_L$ attenuated GA-induced cell death in Caki cells. Furthermore, a sub-toxic dose of GA sensitized TRAIL-mediated apoptosis in Caki cells. Pretreatment with z-VAD completely blocked GA plus TRAIL-mediated apoptosis. On the contrary, pretreatment with NAC partially inhibited GA plus TRAIL-induced apoptosis. Our findings suggested that GA induces apoptosis via the downregulation of cFLIP$_L$ and sensitized TRAIL-mediated apoptosis in Caki cells.

Introduction

Gambogic acid (GA) is a constructive component of *Garcinia hurburyi*, a natural compound derived from brownish gamboe resin in Southeast Asia countries (1). Gamboge resin is used in conventional Chinese medicine for the treatment of hemostasis, detoxification and as a parasiticide (2). GA was reported to have multiple functions, such as anti-inflammation, anti-angiogenesis and anti-invasion (3-5). GA also has potent anticancer activity in numerous types of human cancers, such as lung cancer, hepatocellular carcinoma, malignant melanoma, breast carcinoma, and chronic myelogenous leukemia by targeting NF-κB, thioredoxin reductase, Bcl-2, Akt/mTOR signaling pathway, and proteasome, respectively (6-10). Furthermore, it exhibits specific cytotoxic activity on rapidly dividing cancerous cells with no side effects on normal cells (11). Despite its anticancer efficacy, the molecular mechanism of the GA-induced apoptosis in renal cancer cells is unclear.

TRAIL (tumor necrosis factor (TNF)-related apoptosis-inducing ligand) belongs to the TNF superfamily, which can induce apoptosis in a wide variety of tumor cells, but not normal cells (12). Although TRAIL has beneficial effects in selectively killing tumor cells, many cancer cells are resistant to TRAIL (13). The mechanism of TRAIL resistance is unclear but several studies have reported that TRAIL resistance is intimately associated with the overexpression of anti-apoptosis including cellular FADD-like apoptosis regulator (cFLIP), anti-apoptotic Bcl-2 family proteins (e.g., Bcl-2 and Bcl-xL) and inhibitor of apoptosis proteins (IAPs) (13,14). However, a single treatment with TRAIL may not be sufficient for the treatment of malignant tumor cells. Moreover, TRAIL-resistant cancer cells can be sensitized by a TRAIL sensitizer, such as chemotherapeutic drugs and biochemical inhibitors that suppress the expression of anti-apoptosis-associated proteins, indicating that combination of drugs rather than just one drug alone appears to be more effective in cancer therapy. Therefore, the identification of a novel TRAIL sensitizer is important for effective cancer therapy. The aim of this study was to examine the anticancer effects of GA, elucidate the underlying action mechanism of GA, evaluate GA as a sensitizer of TRAIL, and understand the mechanism of the synergy between GA and TRAIL against human renal cancer cells. In the present study, GA was found to induce apoptosis in renal carcinoma (Caki Cells) through the downregulation of cFLIP$_L$. In addition, a GA treatment rendered human renal cancer cells more sensitive to TRAIL.

Correspondence to: Dr Tae-Jin Lee, Department of Anatomy, College of Medicine, Yeungnam University, 170 Hyeonchung-ro, Nam-gu, Daegu 705-717, Republic of Korea
E-mail: tjlee@med.yu.ac.kr

*Contributed equally

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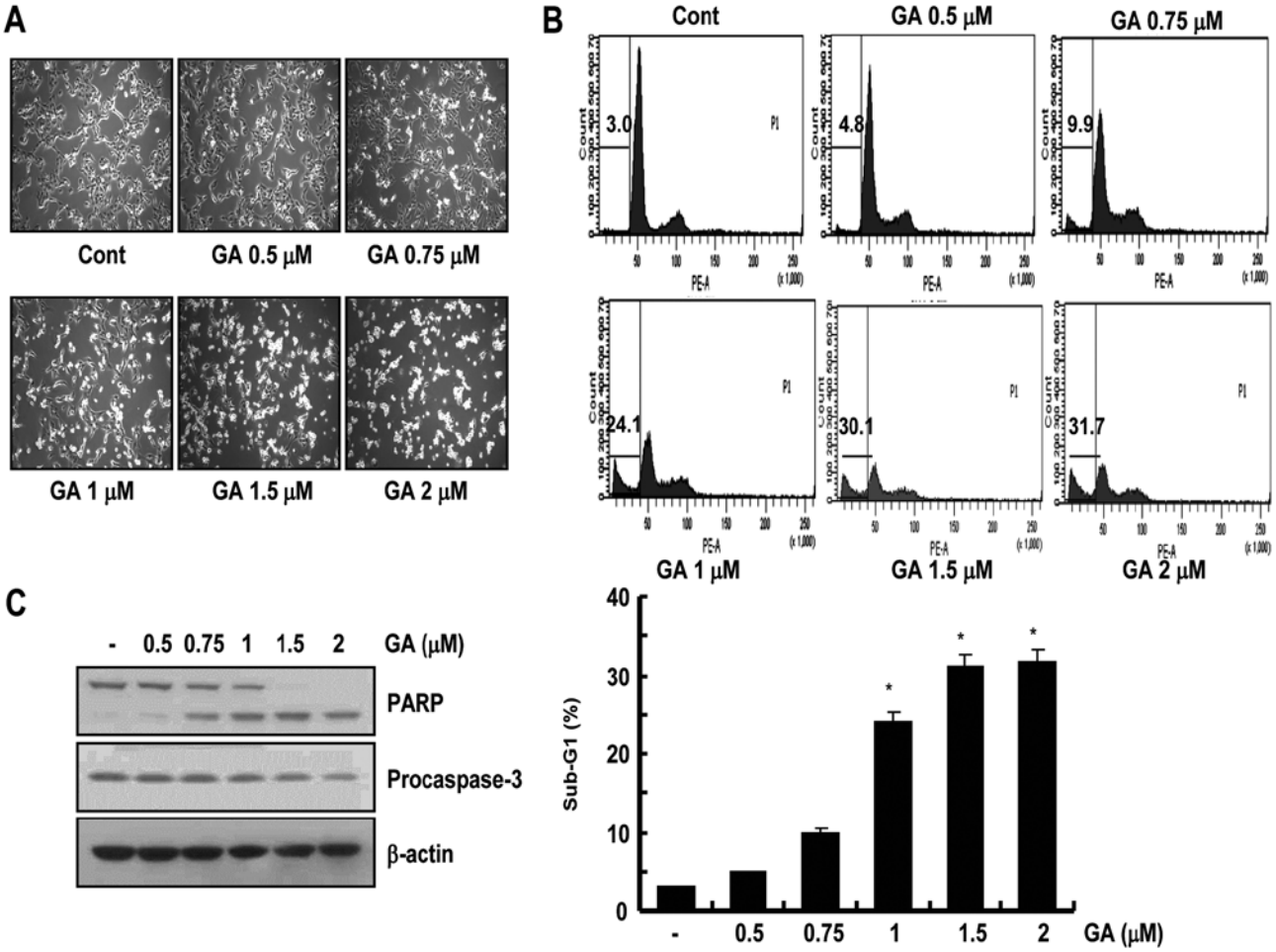

Figure 1. GA induces apoptosis in human renal carcinoma Caki cells. (A) Caki cells were treated with the indicated concentration of GA. After 24 h, the morphological changes were visualized by optical microscopy (magnification, x200). (B) The DNA contents of treated cells were evaluated after propidium iodide staining and apoptosis was measured as a sub-G1 fraction by FACS. The FACS data are shown in the upper panel. The data are reported as the mean values obtained from three independent experiments and the bars represent the standard deviation. $^{*}p<0.05$ compared to the non-treated cells. (C) The cells were treated with the indicated concentrations of GA. Equal amounts of cell lysates (40 μg) were subjected to electrophoresis and analyzed by western blotting for PARP, procaspase-3, and actin for normalization.

Materials and methods

Cells and materials. Caki cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Dulbecco's modified Eagle's medium, containing 10% fetal bovine serum (FBS), 20 mM HEPES buffer and 100 μg/ml gentamicin was used as the culture medium in these experiments. PCR primers were purchased from Bioneer (Daejeon, Korea). The anti-Bcl-2, anti-Mcl-1 and anti-cIAP-2 were acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti $cFLIP_L$ antibody was obtained from ALEXIS Corp. (San Diego, CA, USA). Anti-PARP, and anti-caspase-3 antibody were purchased from Cell Signaling Technology (Beverly, MA, USA). The anti-XIAP antibody was supplied by R&D systems (Minneapolis, MN, USA). Gambogic acid and the other chemicals were purchased from Sigma (St. Louis, MO, USA). Recombinant human TRAIL was obtained from KOMA Biotech (Seoul, Korea).

Cell count and flow cytometry analysis. The cell counts were performed using a hemocytometer. Approximately $0.5x10^6$ Caki cells were suspended in 100 μl of PBS, and 200 μl of 95% ethanol was 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 BD FACSCanto II flow cytometer (BD Biosciences, San Jose, CA, USA) to determine the relative DNA content based on the red fluorescence.

Western blot analysis. The cellular lysates were prepared by suspending $1.2x10^6$ 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 phenylmethylsulfonyl fluoride, and 20 μM leupeptin, adjusted to pH 7.2). The cells were disrupted by sonication and extracted at 4˚C for 30 min. The proteins were electro-transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA, USA). The detection of specific proteins was carried out using an ECL Western blotting kit according to the manufacturer's instructions.

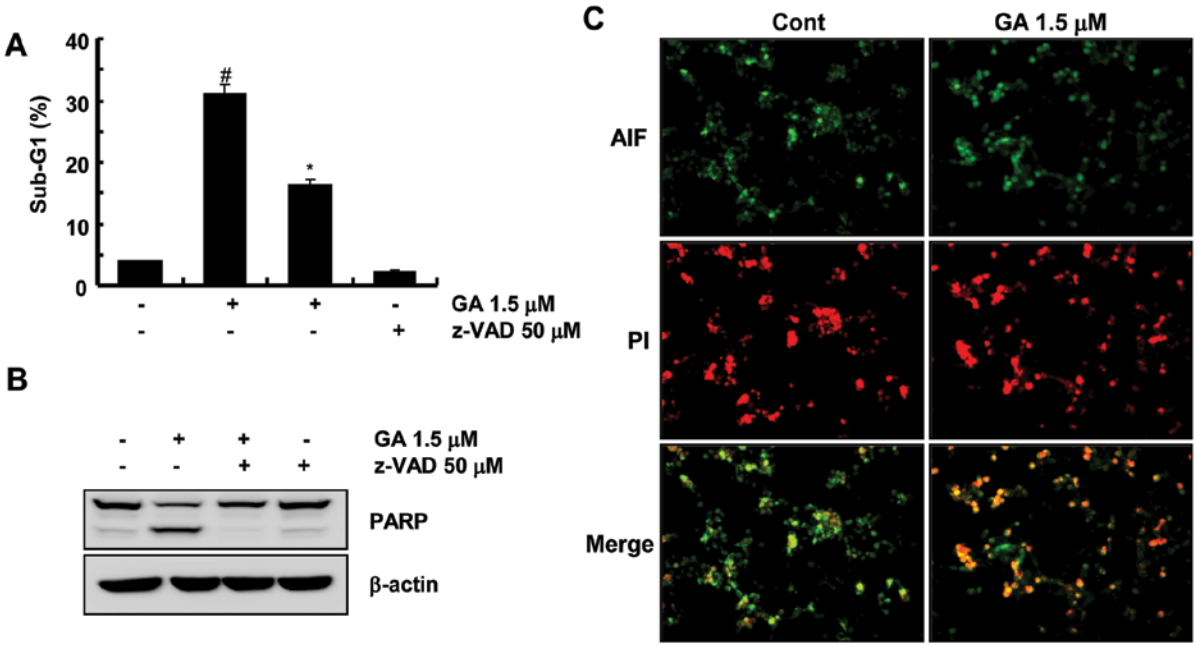

Figure 2. GA-induced apoptosis is partly mediated by caspase-dependent pathway. (A) Caki cells were treated with 1.5 μM GA for 24 h in the presence or absence of 50 μM z-VAD-fmk (z-VAD). Apoptosis was analyzed by determining the sub-G1 cell fraction by FACS. (B) Caki cells were treated with 1.5 μM GA for 24 h in the presence or absence of 50 μM z-VAD-fmk (z-VAD). Equal amounts of cell lysates (40 μg) were subjected to electrophoresis and analyzed by western blotting for PARP. β-actin was used as a control for the protein loading. (C) Immunocytochemistry of the cells with GA treatment for 9 h was performed to localize AIF using an anti-AIF antibody and a FITC-conjugated secondary antibody. The nuclei were stained with PI. Yellow, nuclear translocation of AIF is shown by the overlap of AIF (green fluorescence) and nuclear staining (red fluorescence).

Transfection. For transfection, the cells were plated onto 6-well plates at a density of $0.5x10^6$ cells/well and grown overnight. The cells were co-transfected with 2 μg of various plasmid constructs and 1 μg of pCMV-β-galactosidase plasmid for 5 h using the Lipofectamine method. After transfection, the cells were cultured in 10% FBS medium with a vesicle (DMSO) or drug for 24 h.

RNA isolation and reverse transcriptase-PCR. The expression of $cFLIP_L$ mRNA was determined by RT-PCR. The total cellular RNA was extracted from the cells using an easyBlue reagent (Life Technologies, Seongnam, Korea), and the cDNA was prepared using M-MLV reverse transcriptase (Gibco-BRL, Gaithersburg, MD, USA), according to the manufacturer's instructions. The cellular RNA sample was reverse-transcribed with a random primer and then amplified by PCR, the GAPDH primer set was used as the internal control. The following primers were used to amplify the $cFLIP_L$ and GAPDH. The sequences of the sense and antisense primer for $cFLIP_L$ were 5'-CGG ACT ATA GAG TGC TGA TGG-3' and 5'-GAT TAT CAG GCA GAT TCC TAG-3', respectively. The sequences of the sense and antisense primer for GAPDH were 5'-AGG TCG GAG TCA ACG GAT TTG-3' and 5'-GTG ATG GCA TGG ACT GTG GT-3', respectively. The PCR products were analyzed by electrophoresis on a 1.5% agarose gel and detected by UV light.

4',6'-Diamidino-2-phenylindole (DAPI) staining for nuclei condensation and fragmentation. The cells were fixed with 1% paraformaldehyde on a slide glass for 30 min at room temperature. After washing with PBS, 300 nM 4',6'-diamidino-2-phenylindole (Roche, Mannheim, Germany) was added to the fixed cells for 5 min, and the cells were examined by fluorescence microscopy.

Statistical analysis. The data were analyzed by a one-way ANOVA followed by post-hoc comparisons (Student-Newman-Keuls) using the Statistical Package for Social Sciences 8.0 (SPSS Inc., Chicago, IL, USA).

Results

GA induces apoptosis in renal carcinoma caki cells. To examine the anti-effects of GA in human renal cancer cells, Caki cells were treated with various concentrations of GA. With increasing GA concentration, the GA-treated Caki cells progressively showed the typical features of apoptosis, including cell shrinkage, rounding and detachment of the cell from the plate (Fig. 1A). Cell death was next determined by flow cytometry analysis to detect the hypodiploid cell populations. As shown in Fig. 1B, treatment of Caki cells with GA resulted in a significant increase in the accumulation of sub-G1 phase cells in a dose-dependent manner. In addition, the treatment of Caki cells with GA strongly led to a reduction of the protein levels of 32-kDa precursor together with the concomitant cleavage of PARP, a substrate protein of caspases (Fig. 1C).

GA-induced apoptosis is mediated partly by the AIF translocation. This study next examined whether the activation of caspase pathway plays a critical role in GA-induced apoptosis. As shown in Fig. 2A, GA-mediated apoptosis was prevented

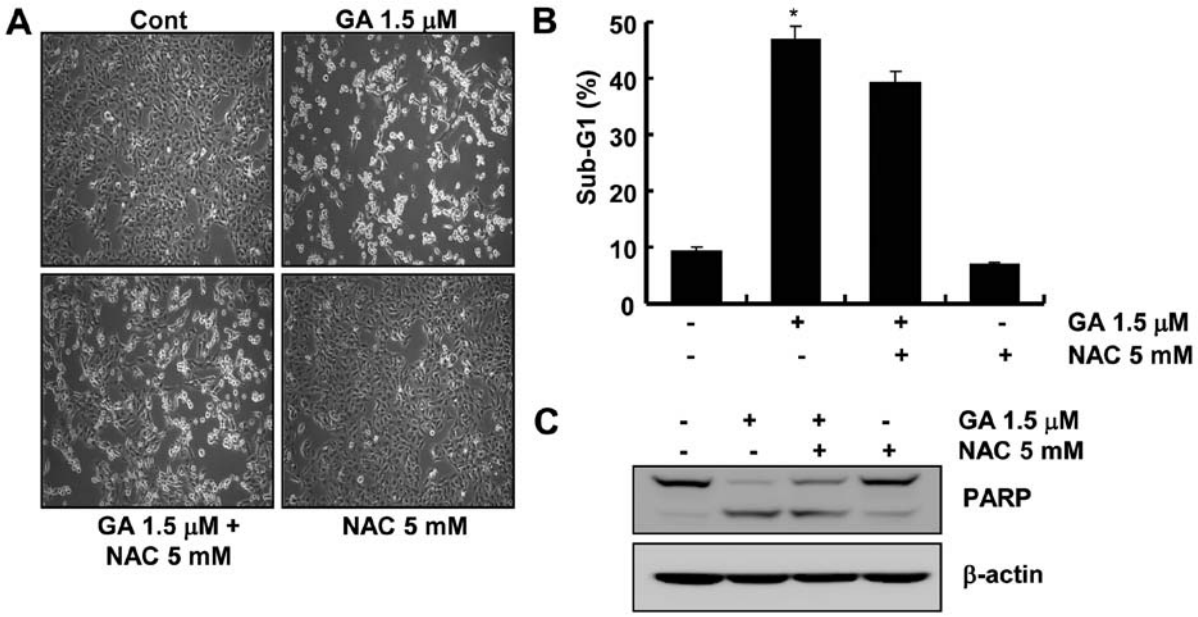

Figure 3. Pretreatment with NAC does not prevent GA-induced apoptosis. (A) Caki cells were treated with 1.5 μM GA for 24 h in the presence or absence of 5 mM NAC. After 24 h, the morphological changes were visualized by optical microscopy (magnification, x200). (B) Caki cells were pretreated with 5 mM NAC for 30 min, and then stimulated with 1.5 μM GA for 24 h. Apoptosis was analyzed by determining the sub-G1 cell fraction by FACS. (C) Equal amounts of cell lysates (40 μg) were subjected to electrophoresis and analyzed by western blotting for PARP. β-actin was used as a control for the protein loading.

partly by a pretreatment with a general and potent inhibitor of caspases, z-VAD-fmk. In contrast, treatment with z-VAD-fmk completely prevented these caspase-related events, such as the cleavage of PARP (Fig. 2B). These results suggest that the GA-induced cell death was mediated partly by the caspase-dependent pathway and caspase-independent cell death in the presence of z-VAD-fmk. Because AIF is involved in the induction of apoptotic cell death through the caspase-independent pathway, this study examined whether AIF plays a role in GA-induced apoptotic cell death. The translocation of AIF was analyzed by the observation of its release from the mitochondria and translocation to the nucleus by fluorescence microscopy. As shown in Fig. 2C, fluorescence microscopy showed that AIF was translocated to the nucleus and caused nuclear condensation after the treatment with GA. This suggests that GA-induced apoptotic cell death in Caki cells is mediated by AIF translocation from the mitochondria into the nucleus via a caspase-independent pathway.

Pretreatment with N-acetylcysteine (NAC) does not affect GA-induced apoptosis. Further experiments were conducted to determine if ROS generation induced by GA is associated directly with the induction of apoptosis because numerous studies have documented that GA induces apoptosis through ROS accumulation in human cancer cells (15,16). On the contrary, as shown in Fig. 3A and B, pretreatment with N-acetylcysteine (NAC), a ROS scavenger, did not prevent GA-induced cell death. In addition, pretreatment with NAC failed to prevent the cleavage of PARP (Fig. 3C). These results suggest that ROS generation is not critical for the induction of apoptosis by GA.

Downregulation of cFLIP$_L$ contributes to GA-induced apoptosis. This study next examined whether the downregulation of cFLIP$_L$ is critical to stimulate GA-induced apoptosis. The overexpression of cFLIP$_L$ in Caki cells attenuated GA-mediated apoptosis, whereas treatment with GA-induced apoptosis in Caki/vector cells (Fig. 4C). This suggests that cFLIP$_L$ downregulation also contributes to GA-induced apoptosis. The cleavage of PARP and procaspase-3 was also analyzed in both cell lines. As shown in Fig. 4D, the ectopic expression of cFLIP$_L$ attenuated the cleavage of PARP and procaspase-3 induced by GA treatment in Caki cells. The levels of cFLIP$_L$ and cFLIPs expression were checked after a treatment with various concentration of GA to determine if GA specifically downregulates cFLIP$_L$ expression without affecting the levels of cFLI$_P$s. The treatment with GA decreased the level of cFLIP$_L$ expression in Caki cells. In contrast, no changes in the cFLIPs mRNA and protein levels were detected in the GA-treated cells (Fig. 4E and F). These results suggest that GA suppressed cFLIP$_L$ expression with little effect on cFLIP expression in the present system. Interestingly, we found that GA treatment induced the cleavage of p65 protein, a subunit of nuclear factor-κB (NF-κB), subsequently reduction of p65 protein level in Caki cells (Fig. 4G). This result suggested the possibility that GA-induced cFLIP$_L$ downregulation might be caused by inhibiting NF-κB pathway in our system.

GA sensitizes renal cancer cells for TRAIL-mediated apoptosis. In an attempt to search for novel strategies to overcome TRAIL resistance in cancer cells, this study examined the effects of a combination treatment of GA and TRAIL in Caki cells. The co-treatment of Caki cells with GA and TRAIL resulted in a marked increase in the accumulation of sub-G1 phase cells compared to Caki cells treated with GA or TRAIL alone (Fig. 5A). In addition, a combined treatment with GA and TRAIL induced chromatin condensation paralleled by nuclear fragmentation (Fig. 5B). Finally, the combinational treatment

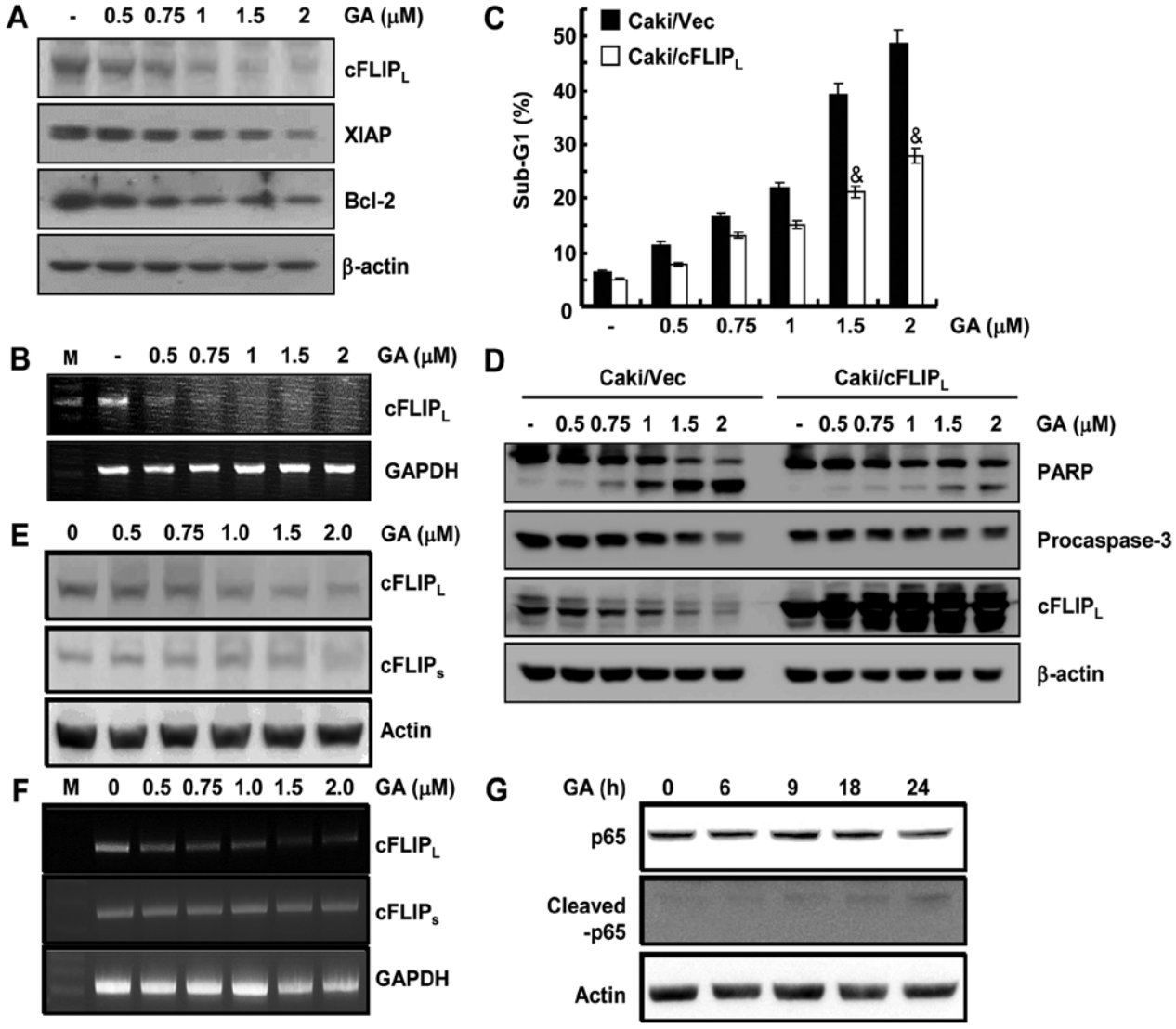

Figure 4. Downregulation of $cFLIP_L$ contributes to GA-induced apoptosis. (A) Caki cells were treated with the indicated concentrations of GA. Equal amounts of cell lysates (40 μg) were subjected to electrophoresis and analyzed by western blotting for $cFLIP_L$, XIAP, Bcl-2, and actin for normalization. (B) Caki cells were treated with the indicated concentrations of GA for 24 h. The 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. (C) Caki/vector and Caki/$cFLIP_L$ cells were treated for 24 h with the indicated concentrations of GA and then evaluated for the DNA content after propidium iodide staining. Apoptosis was analyzed as a sub-G1 fraction by FACS. (D) The Caki/vector and Caki/cc$FLIP_L$ cells treated as above were harvested in a lysis buffer and equal amounts of cell lysates (40 μg) were subjected to electrophoresis and analyzed by western blotting for PARP, procaspase-3 and cFLIP. β-actin was used as a control for the protein loading. (E) Caki cells were treated with the indicated concentrations of GA. Equal amounts of cell lysates (40 μg) were subjected to electrophoresis and analyzed by western blotting for cFLIP and actin for normalization. (F) Caki cells were treated with the indicated concentrations of GA for 24 h. The total RNA was isolated and RT-PCR analysis was performed, as described in Materials and methods. (G) Caki cells were treated with 1.5 μM of GA during the indicated times. Equal amounts of cell lysates (40 μg) were subjected to electrophoresis and analyzed by western blotting for cFLIP and actin for normalization.

of Caki cells with GA and TRAIL led to a decrease in the protein levels of procaspase-3 with the concomitant cleavage of PARP protein (Fig. 5C).

Combination treatment with GA plus TRAIL-induced apoptosis is mediated via caspase-dependent pathway. This study next examined whether activation of the caspase pathway plays a critical role in GA plus TRAIL-induced apoptosis. As shown in Fig. 5D, GA plus TRAIL-induced apoptosis was prevented by a pretreatment with z-VAD-fmk, as determined by FACS analysis. In addition, z-VAD-fmk prevented these caspase-related events including the cleavage of procaspase-3 and PARP (data not shown). These results suggest that a combined treatment of GA and TRAIL-induced apoptosis was mediated by caspase-dependent apoptosis pathways. Furthermore, NAC partly prevented GA plus TRAIL-mediated apoptosis in Caki cells (Fig. 5E).

Discussion

In the present study, GA exhibited significant antitumor activity against human renal cancer cells. GA-induced apoptosis in human Caki renal cancer cells was mediated by two different pathways (e.g., caspase-dependent and caspase-independent

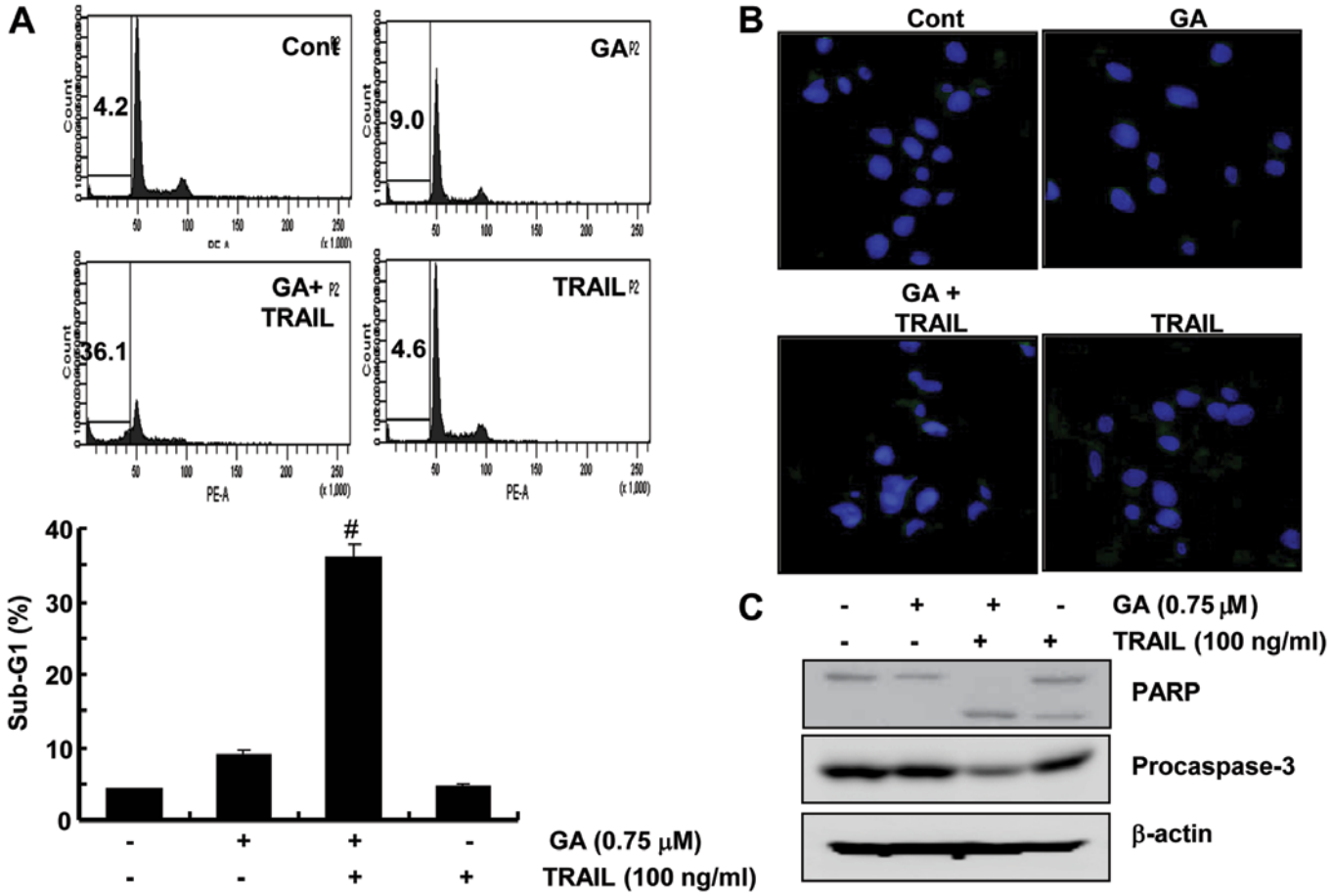

Figure 5. Gambogic acid sensitized TRAIL-mediated apoptosis in renal carcinoma Caki cells. (A) Caki cells were treated with 100 ng/ml of TRAIL in the presence or absence of 0.75 μM GA for 24 h. The DNA contents of the treated cells were evaluated after propidium iodide staining and apoptosis was measured as a sub-G1 fraction by FACS. The FACS data is shown in the upper panel. The data are reported as the mean values obtained from three independent experiments and the bars represent the standard deviation. $^{*}p<0.05$ for GA+TRAIL-treated cells versus control, GA-, or TRAIL-treated cells by ANOVA. (B) After treatment with GA plus and TRAIL for 24 h, the cells were harvested and washed with PBS and exposed to 300 nM 4',6-diamidino-2-phenylindole (DAPI) at room temperature in the dark for 15 min. The samples were observed by fluorescence microscopy. (C) Caki cells treated as above were harvested in lysis buffer and equal amounts of the cell lysates (40 μg) were subjected to electrophoresis and analyzed by western blotting for PARP and procaspase-3. β-actin was used as a control for the protein loading.

pathways). In addition, GA-induced apoptosis was mediated by the downregulation of $cFLIP_L$ expression, and the treatment of renal cancer cells with GA in combination with TRAIL synergistically induced apoptosis.

Natural bioactive compounds have attracted considerable attention as chemotherapeutic agents over the past year because of their safety and efficacy in overcoming tumor cell growth by inducing apoptosis by upregulating the pro-apoptotic proteins or downregulating anti-apoptotic proteins (17-20). GA is a natural compound of *Garcinia hurburyi*, which has been used for medicinal purpose in Southeast Asia countries since ancient times (21). As GA has anticancer effects on cancer cells with very weak activity on the hematologic system, it has been approved for phase II clinical trials for solid cancer therapy (10,23). In addition, caspase activation is required for GA-induced apoptotic cell death in cancer cells (10,23). In the present study, GA activated the caspase-dependent apoptotic pathway in a dose-dependent manner in Caki cells, which was partly prevented by pretreatment with the pancaspase inhibitor, z-VAD-fmk. This suggests that GA-induced apoptosis is mediated by caspase-independent and caspase-dependent apoptotic pathways. To determine which type of cell death is induced by GA, the caspase-independent cell death machinery involved in the alternate pathway activated by GA was first defined. AIF mediates cell death through a caspase-independent pathway. Mitochondrial AIF translocates to the nucleus on death stimuli and initiates nuclear condensation, which leads to large-scale chromatin fragmentation followed by cell death (24,25). Fluorescence microscopy revealed the translocation of AIF into the nucleus of Caki cells, suggesting that the activation of caspase-independent apoptotic route is mediated by the translocation of AIF into the nucleus.

Reactive oxygen species (ROS) are used as active mediators for the regulation of cell death, including caspase-dependent and caspase-independent pathways and necrosis (26,27). In addition, exposure of human bladder cancer cells to GA induces an increase in ROS accumulation, which causes caspase activation, finally leading to cell death (28). In the present study, GA treatment was found to elicit ROS generation (data not shown), but the inhibition of ROS by NAC did not prevent GA-induced apoptosis in Caki cells. This suggests that ROS generation is not involved in GA-mediated cell death in the present system.

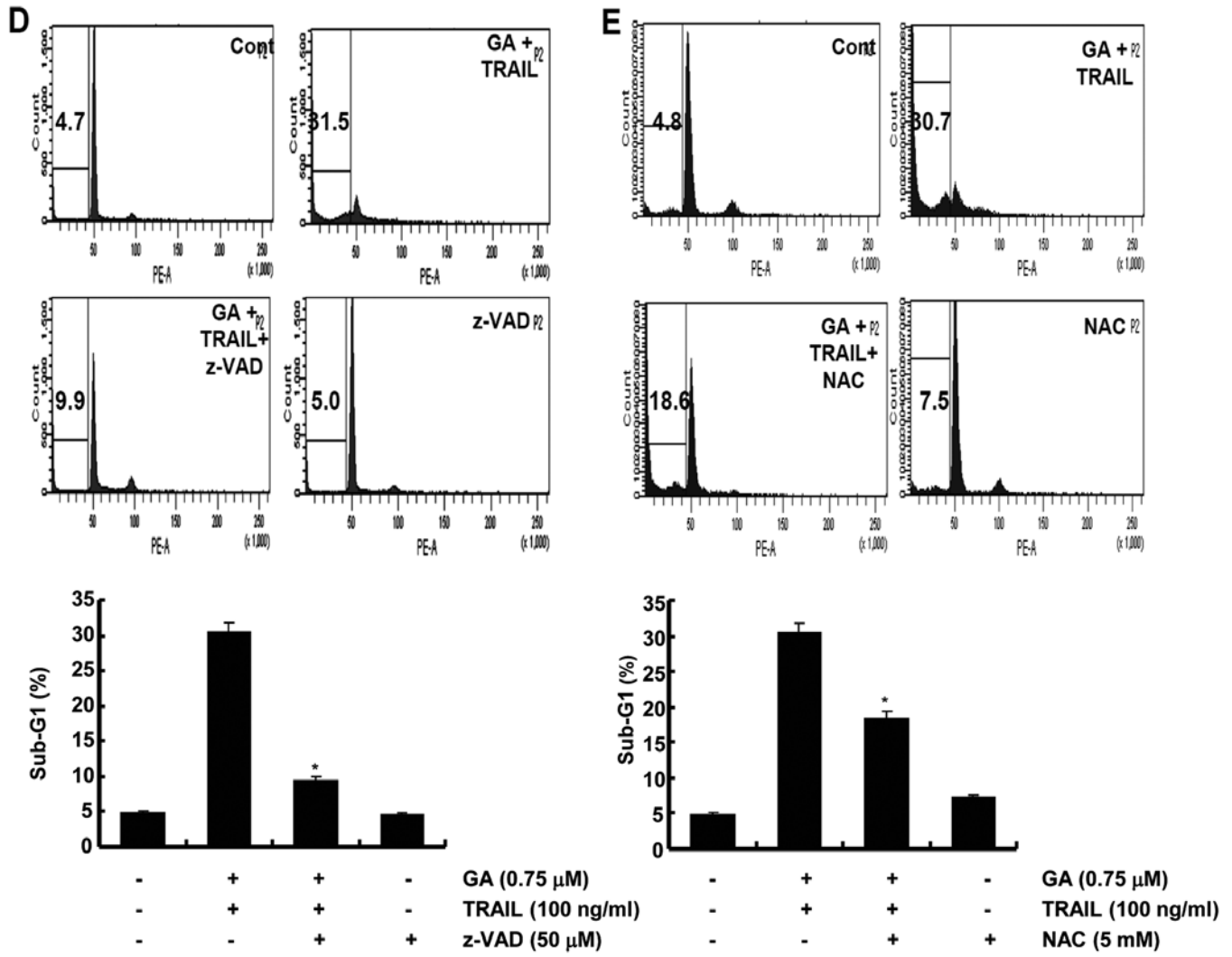

Figure 5. Continued. (D) Caki cells were incubated with 50 μM z-VAD-fmk or solvent for 1 h before the treatment with GA and/or TRAIL (100 ng/ml) for 24 h. The DNA contents of the treated cells were evaluated after propidium iodide staining and apoptosis was measured as a sub-G1 fraction by FACS. The data are reported as the mean values obtained from three independent experiments and the bars represent the standard deviation. *p<0.05 compared to GA plus TRAIL-treated cells. (E) Caki cells were pretreated with 5 mM NAC for 30 min, and then stimulated with GA and/or TRAIL (100 ng/ml) for 24 h. Apoptosis was analyzed by determining the sub-G1 cell fraction by FACS. The data are reported as the mean values obtained from three independent experiments and the bars represent the standard deviation. *p<0.05 compared to the GA plus TRAIL-treated cells.

GA has a strong cytotoxic effect on a variety of cancers via repressing the NF-κB pathways, downregulating Bcl-2 expression and inhibiting the proteasome pathways (29-31). Consistent with previous findings, GA treatment resulted in downregulation of Bcl-2 proteins in renal cancer cells. Interestingly, we found that GA downregulated $cFLIP_L$ expression by suppressing transcriptional control as well as that GA induced cleavage of p65 protein in Caki cells. It is generally recognized that $cFLIP_L$ protein levels can be transcriptionally regulated through the NF-κB (32,33). Based on these previous studies and our finding, we postulated the possibility that GA downregulated cFLIP mRNA expression via suppressing NF-κB pathway in our system. Furthermore, the ectopic expression of $cFLIP_L$ attenuated GA-induced cell death, suggesting that $cFLIP_L$ downregulation is responsible for the GA-induced apoptotic cell death in the present system.

The cFLIP gene often generates two splicing isoforms, $cFLIP_L$ and cFLIPs, by alternative splicing in humans. $cFLIP_L$ and cFLIPs play different roles in cell death, including apoptosis, necroptotic cell death and autophagy (34-37). Therefore, expression of the cFLIP isoforms would need to be regulated specifically for cancer therapy. In the present study, GA specifically downregulated $cFLIP_L$ mRNA expression with no marked change in the cFLIPs mRNA levels. The $FLIP_L$ protein can also block the recruitment of caspase-8 to DISC, suppressing death receptor-mediated apoptosis (38). Previous studies showed that GA could markedly sensitize chemotherapeutic drug-induced cell death in a range of cancer cells (6,39-41). Therefore, based on these studies, this study examined whether GA-induced $cFLIP_L$ downregulation could increase the sensitivity to the death ligands, including TRAIL, TNF-α and FasL in human renal cancer cells. This study discovered that GA increased the sensitivity to TRAIL, suggesting that GA is a sensitizer of the TRAIL-mediated apoptotic pathways in renal cancer cells.

In conclusion, we suggest that GA plays an important role as a proapoptotic agent via the specific downregulation of $cFLIP_L$ expression without affecting the expression of cFLIPs.

In addition, GA treatment rendered human renal cancer cells more sensitive to TRAIL. These results suggested that a combined treatment of GA and TRAIL may provide a safe and effective therapeutic strategy against cancers that are resistant to conventional treatments. Furthermore, this study provided novel evidence that the prominent sensitizing effect of GA on TRAIL-induced apoptosis is due to the downregulation of $cFLIP_L$.

Acknowledgements

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