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Chlorogenic Acid Induces Apoptotic Cell Death in U937 Leukemia Cells through Caspase- and Mitochondria-dependent Pathways

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Abstract. *Chlorogenic acid exists widely in edible and medicinal plants and acts as an antioxidant. It is known to exert antitumor activity via induction of apoptosis in many human cancer cells. However, its signaling pathway in human leukemia cells still remains unclear. Therefore, we investigated the roles of reactive oxygen species (ROS), mitochondria and caspases during chlorogenic acid-induced apoptosis of U937 human leukemia cells. Chlorogenic acid exhibited a strong cytotoxicity and induced apoptosis in U937 cells, as determined by 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) staining and terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay. Chlorogenic acid induced apoptosis by promoting ROS production and reduced the mitochondrial membrane potential ($\Delta\Psi m$), as assayed by flow cytometry. Furthermore, the activity of caspase-3 was evaluated and results indicated that chlorogenic acid promoted caspase-3 activity in U937 cells. Results from western blot analysis showed that chlorogenic acid promoted expression of caspase-3, -7, -8 and -9 in U937 cells. Taken together, these results suggest that chlorogenic acid may induce apoptosis by reducing the levels of $\Delta\Psi m$ and by increasing the activation of caspase-3 pathways in human leukemia U937 cells in vitro.*

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Development of drug resistance in tumor cells and side-effects in patients have led to limitations to current chemotherapy in patients with leukemia (1, 2). In clinical practice, camptothecin from *Camptotheca acuminata* and paclitaxel from Taxus brevifolia, originating from natural products, are currently used as chemotherapeutic agents (3); both compounds can induce cell cytotoxic effects, including the induction of cell-cycle arrest and apoptosis (4, 5).

It is well-known that caspases (a group of cysteine proteases) play important roles in apoptosis. After injury to mitochondria, cytochrome *c* and other apoptotic-inducing factors can be released from mitochondria and then also activate caspase-3, -7 or -9 signals (6, 7). Thus, agents which can induce caspase activation may lead to the induction of apoptosis and it has been recognized that induction of cancer

cell apoptosis is the best strategy for blocking cancer development (8-10).

Chlorogenic acid, a dietary polyphenol with a long history of use in Chinese medicine, exists widely in edible and medicinal plants (11). Chlorogenic acid has been reported to have antioxidant activities (12), and is beneficial in oxidative stress-related diseases (13-16). It has also anti carcinogenic activities (16), including induction of apoptosis in human oral squamous cell carcinoma and salivary gland tumor cell lines (17), and BCR^{-}-ABL^{+} chronic myeloid leukemia (CML) cells (18). However, there is no report regarding chlorogenic acid-induced apoptosis in leukemia cells. The present study investigated the cytotoxic effects of chlorogenic acid on U937 human myelocytic leukemic cells.

Materials and Methods

Chemicals and reagents. Chlorogenic acid, dimethyl sulfoxide (DMSO), propidium iodide (PI), Tris-HCl, Triton X-100 and trypan blue were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). RPMI-1640 medium, L-glutamine, fetal bovine serum (FBS), penicillin-streptomycin, trypsin-EDTA and 4,6-diamidino-2-phenylindole dihydrochloride (DAPI), were obtained from Gibco Life Technologies (Grand Island, NY, USA). The caspase-3 substrate kit Ac-Asp-Glu-Val-Asp-chromophore *p*-nitroaniline (Ac-DEVD-pNA) was obtained from R&D Systems Inc. (Minneapolis, MN, USA).

Cell culture. The U937 human myelocytic leukemic cell line was from the Food Industry Research and Development Institute (Hsinchu, Taiwan, ROC). U937 cells were placed in 75-cm^2 culture flasks and were grown in RPMI-1640 supplemented with 10% FBS, 100 U/ml penicillin-100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2 and 95% air at 37˚C (19).

Determination of cell viability and morphology. U937 cells were plated at a density of 5×10^5 cells/well in a 12-well plate for 24 h, then were incubated with 0, 50, 100, 150 and 200 μM of chlorogenic acid at 37˚C, with 5% CO_2 and 95% air for 48 h. Cells were examined and photographed by phase-contrast microscopy for the examination of morphological changes. Then cells were harvested by centrifugation and were stained with PI (4 μg/ml) then analyzed by flow cytometry (FACSCalibur flow cytometer; BD Biosciences, San Jose, CA, USA) for viability measurements, as previously described (19, 20).

DAPI staining. For DAPI staining, approximately 5×10^4 U937 cells/ml were treated with 0, 100, 150 and 200 μM of chlorogenic acid for 48 h. Cells in each well were stained with DAPI, then examined and photographed using a fluorescence microscope as previously described (21, 22).

Terminal deoxynucleotidyl transferase dUTP nick-end labeling staining. For TUNNEL staining, approximately 5×10^4 cells/ml of U937 cells were treated with 0, 100, 150 and 200 μM of chlorogenic acid for 48 h. Cultured U937 cells were recovered and were attached to coverslips covered with poly-L-lysine and fixed with 4% paraformaldehyde (PFA) for 10 min at room temperature, then were washed with phosphate-buffered saline (PBS). Then the samples were mounted with Vectashield (Vector Laboratories Inc., Burlingame, CA, USA) and examined under an Olympus BH2 epifluorescense microscope (Olympus, Tokyo, Japan). The TUNEL-positive cells were quantified in random fields as a percentage of the total number of U937 cells in the field (23, 24).

Caspase-3 activity assay. Caspase-3 activity was measured through the absorbance at 405 nm after cleavage of synthetic substrate Ac-DEVD-pNA. U937 cells were plated at a density of 5×10^5 cells/well in T75 flasks for 24 h, and were then incubated with 0, 50, 100, 150 and 200 μM of chlorogenic acid at 37˚C, with 5% CO_2 and 95% air for 48 h. At the end of the incubation, cells were collected and lysed on ice for 30 min in the cell lysis buffer with the R&D system colorimetric assay kit. The lysates (50 mg) were reacted with 50 mM Ac-DEVD-pNA in a reaction buffer (1% NP-40, 20 mM Tris–HCl, 137 mM NaCl, 10% glycerol, 10 mM dithiothreitol, and protease inhibitors, pH 7.4), then the mixtures were maintained at 37°C for 2 h and subsequently analyzed in an enzyme-linked immunosorbent assay reader (Molecular Devices). The enzyme activity was calculated on the basis of a standard curve prepared using *p*-nitroanaline (pNA), as described previously (19, 25). The relative levels of pNA were normalized against the protein concentration under each treatment.

Detection of reactive oxygen species (ROS) and mitochondrial membrane potential ($\Delta\Psi m$). U937 cells were placed at a density of 5×10^5 cells/well in a 12-well plate for 24 h, then were incubated with 0, 50, 100, 150 and 200 μM of chlorogenic acid at 37˚C with 5% CO_2 and 95% air for 12 h. Cells from each treatment were harvested and re-suspended in 500 μl of 10 μM (DCFH-DA; 2,7- dichlorodihydro-fluorescein diacetate) for ROS and in 500 μl of 1 μM dihexyloxacarbocyanine iodide ($DiOC_6$) for $\Delta\Psi m$. Cells were incubated at 37˚C for 30 min before being analyzed by flow cytometry, as described previously (21, 22).

Western blotting of apoptosis-associated proteins. Approximately 1×10^7 cells of U937 cells in 6-well plates were then treated with 0, 50, 100, 150 and 200 μM of chlorogenic acid for 48 h. Cells were harvested and lysed with lysis buffer (PRO-PREP™ protein extraction solution, iNtRON Biotechnology, Seongnam-si, Gyeonggi-do, Korea). The total proteins from each treatment were quantified and 30 μg were used for western blot analysis and all samples were analyzed using 10% Tris-glycine-SDS-polyacrylamide gels for 30 min and then the proteins were transferred to a nitrocellulose membrane by electroblotting, as described previously (19-22). The membranes were stained with primary antibodies against caspase-3, -8, -9 and -7 (R&D Systems) then were washed and incubated with a secondary antibody for enhanced chemiluminescence (Immobilon Western HRP substrate, Merck Millipore, Bedford, MA, USA), as described previously (21, 26).

Results

Chlorogenic acid reduces cell viability and induces morphological changes of U937 cells. After exposure to 0, 50, 100, 150 or 200 μM of chlorogenic acid for 48 h, cells were examined and photographed under phase-contrast microscopy and the results are shown in Figure 1A. The results indicated that cell death in chlorogenic acid-treated

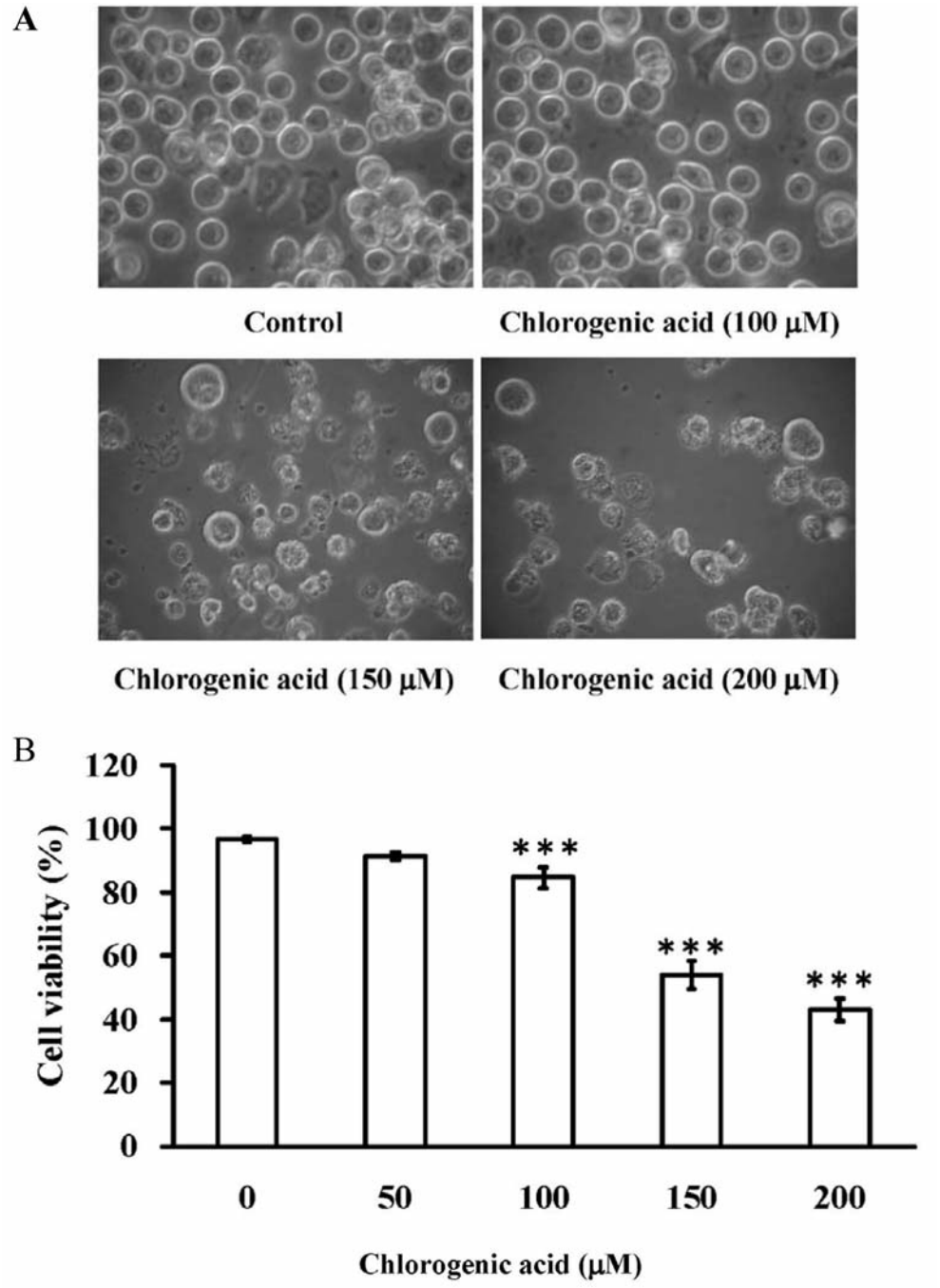

Figure 1. *Chlorogenic acid reduced cell viability and induced cell morphological changes of U937 cells. Cells were incubated with different concentrations of chlorogenic acid for 24 and 48 h. The viability of cells measured (A) and cell morphological changes were examined as described in the Materials and Methods. Each point corresponds to the mean±SD (n=3), ***p<0.001 for the difference between chlorogenic acid, cells treated and control in U937 cell.*

cells was greater, based on the higher level of cell debris compared to those of control, leading to lower cell numbers. These effects were concentration-dependent. The cytotoxic effects of chlorogenic acid on U937 cells were examined by flow cytometric assay and the results are shown in Figure 1B. The results indicated that chlorogenic acid treatment caused a concentration-dependent decrease in the viability of U937 cells.

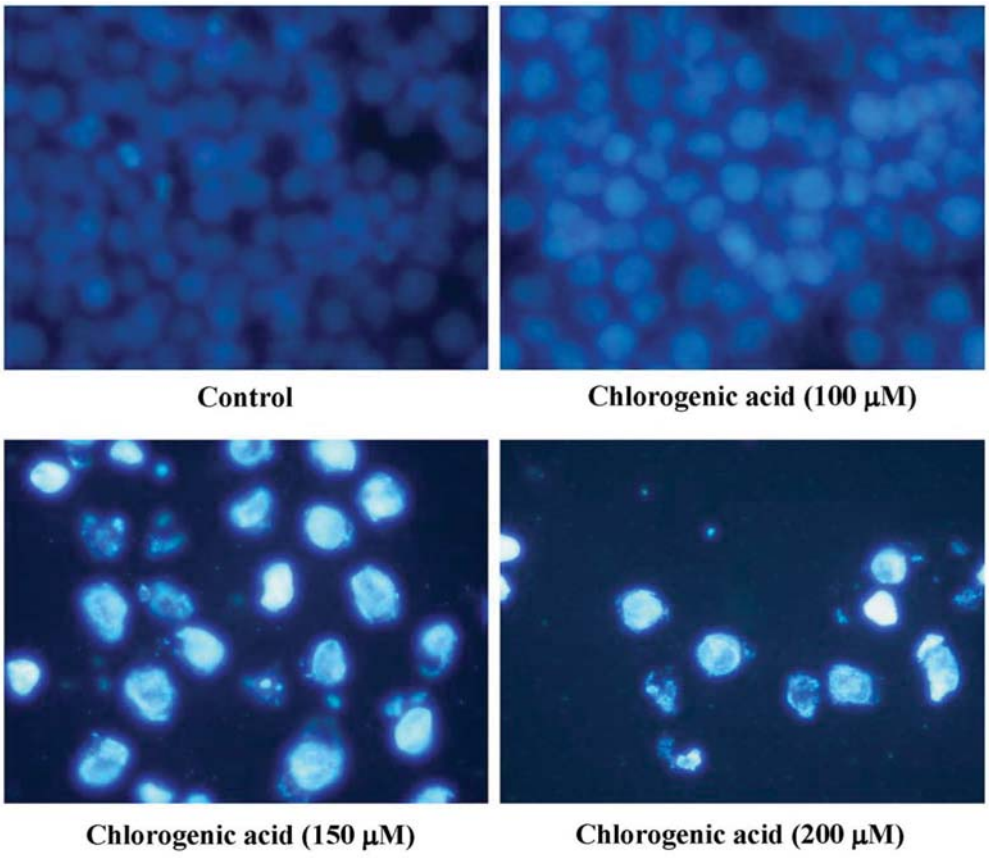

Figure 2. *Chlorogenic acid induced apoptosis in U937 cells. Cells at a density of 1×10^5 cells/well were incubated with different concentrations of chlorogenic acid for 48 h. Cells were stained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) for 30 min, then were examined and photographed under a fluorescence microscope, as described in Materials and Methods.*

Chlorogenic acid induces apoptosis of U937 cells. For further investigating the mode of death of U937 cells after exposure to chlorogenic acid, cells were exposed to chlorogenic acid (0, 100, 150 or 200 μM) for 48 h and were then staining by DAPI for examination of apoptotic cell death. The results are shown in Figure 2, and indicate that chlorogenic acid induced apoptosis based on the higher number of white-colored nuclei in treated cells compared to those of the control.

Chlorogenic acid induces DNA fragmentation (apoptosis) in U937 cells. Cells were exposed to different concentrations (0, 50, 100, 150 or 200 μM) of chlorogenic acid for 24 h, then were stained with TUNEL and photographed and the results are shown in Figure 3. In the TUNEL assay, chlorogenic acid treatment increased the number of cells with DNA strand breaks in a dose-related manner (Figure 3). *Chlorogenic acid induces caspase-3 activation of U937 cells*. Cells were exposed to different concentration of chlorogenic acid for 24 h, then were lysed for measuring the activitiy of caspase-3 by using enzyme-linked immunosorbent assay and the results are shown in Figure 4.

Chlorogenic acid induces ROS production and affects the level of $\Delta\Psi m$ in U937 cells. To confirm whether chlorogenic acid induces apoptosis *via* the mitochondrial pathway, U937 cells were treated with 0, 100, 150 and 200 μM chlorogenic acid for 12 h, and the ROS levels and $\Delta\Psi m$ were measured and determined by flow cytometric assay. As shown in Figure 5A and B, chlorogenic acid treatment of U937 cells led to an increase in the production of ROS (Figure 5A) and also induced a decrease of $\Delta\Psi m$ (Figure 5B). These effects were concentration-dependent.

Chlorogenic acid affects the levels of apoptosis proteins in U937 cells. To investigate whether chlorogenic acid induced apoptosis-involved caspase-associated protein expression, U937 cells were exposed to chlorogenic acid and then cells were harvested for western blotting. As shown in Figure 6, chlorogenic acid promoted the expression of caspase-3, -7, -8

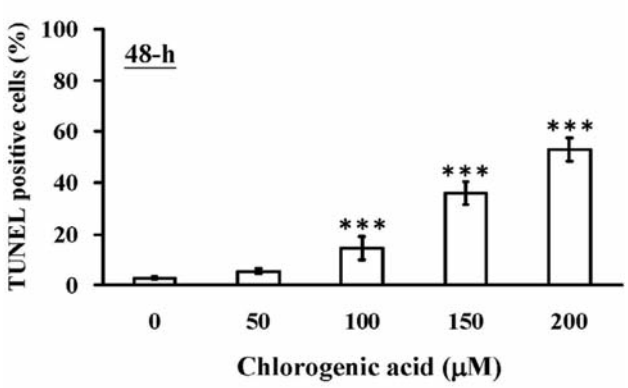

Figure 3. *Chlorogenic acid induced DNA fragmentation (apoptosis) in U937 cells. Cells at a density of 1×10^5 cells/well were incubated with different concentrations of chlorogenic acid for 48 h. Then cells were stained by the TUNEL assay for 30 min then were examined and photographed under a fluorescence microscope, as described in the Materials and Methods. ***p<0.001.*

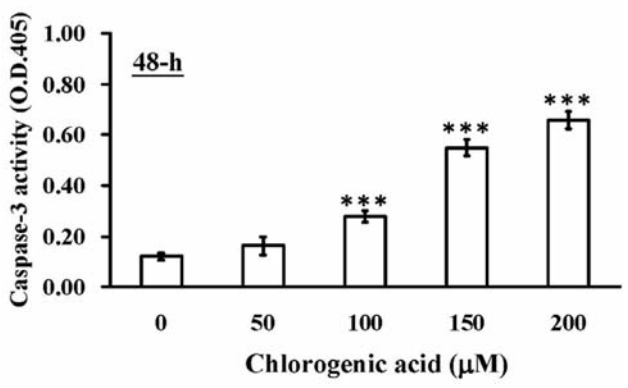

Figure 4. *Chlorogenic acid induced caspase-3 activation in U937 cells. Cells at a density of 1×10^5 cells/well were incubated with different concentrations of chlorogenic acid for 48 h. The cells were lysed and incubated with caspase-3 substrate (Ac-DEVD-pNA) and the activity of caspase-3 was measured by enzyme-linked immunosorbent assay, as described in the Materials and Methods. ***p<0.001 for the difference between chlorogenic acid-treated and control U937 cells.*

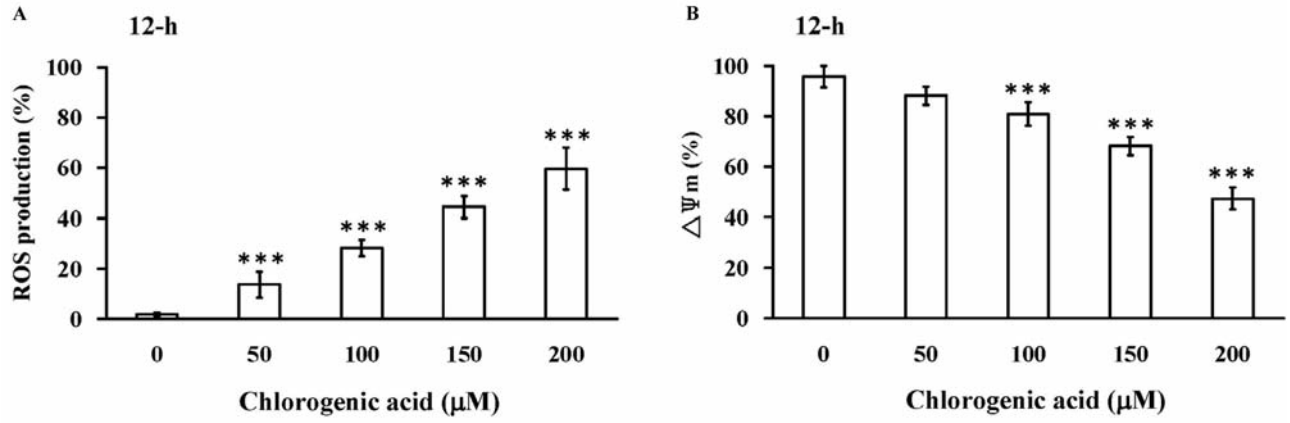

Figure 5. *Chlorogenic acid induced reactive oxygen species (ROS) production and affected the level of mitochondrial membrane potential (ΔΨm) in U937 cells. Cells (2×10^5 cells/ml) were treated with chlorogenic acid for 12 h. Cells were harvested for analysis of ROS (A) and ΔΨm (B), as shown by staining by 2,7- dichlorodihydrofluorescein diacetate (DCFH-DA) and dihexyloxacarbocyanine iodide (DiOC6), respectively. The stained cells were determined by flow cytometry, as described in the Materials and Methods. Values are means±SD (n=3). *Significantly different from that at 0 h treatment (control group) at ***p<0.001.*

and -9 proteins, which suggests that chlorogenic acid induced apoptosis in U937 cells through a caspase-dependent pathway.

Discussion

Natural products such as camptothecin and paclitaxel have been developed as anticancer agents in the clinical setting (27, 28). Chlorogenic acid exists in natural plants and although studies have shown that chlorogenic acid induces cytotoxic effects in many human cancer cells (13-16), the underlying signal transduction pathway in human leukemia cells is still unclear. Therefore, the present study focused on the elucidation of the role of caspases and mitochondria during apoptosis of U937 human leukemia cells, induced by chlorogenic acid. We found that chlorogenic acid had a strong cytotoxicity towards U937 cells in a dose-dependent manner (Figure 1). However, the effective concentration for cytotoxicity of chlorogenic acid towards U937 cells was found to be greater than 100 μM (Figure 1). Furthermore, we also found that caspase-3, -7, -8 and -9 were activated prior to the development of apoptosis in U937 cells exposed to chlorogenic acid (Figure 6).

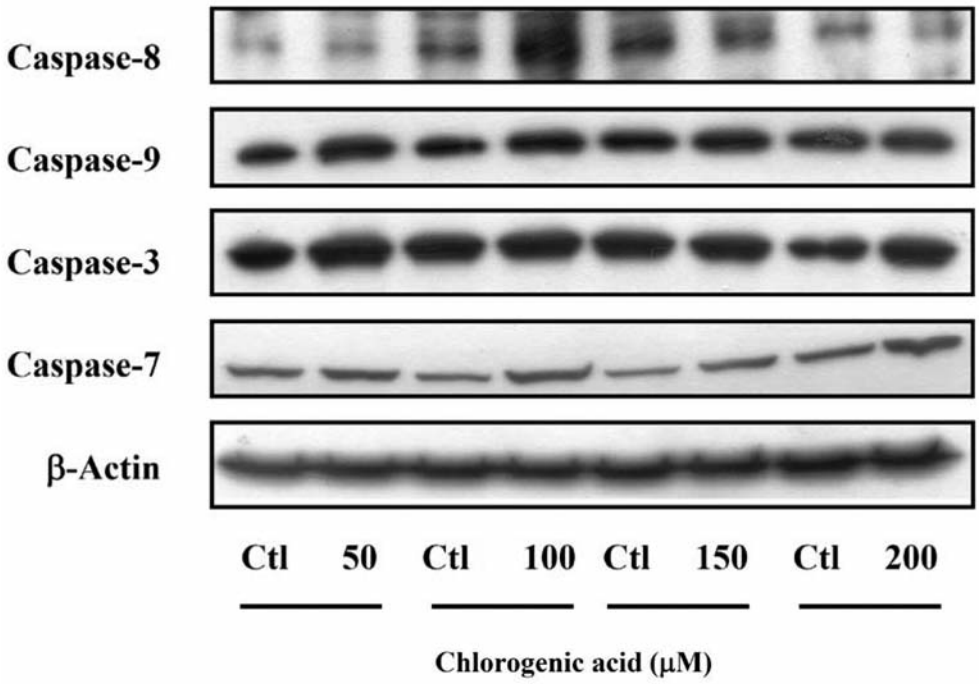

Figure 6. *Chlorogenic acid affects the level of apoptosis-related proteins in U937 cells. Cells were treated with chlorogenic acid for 48 h then the total proteins were prepared and detected by western blotting as described in the Materials and Methods. Primary antibodies for caspase-3, -7, -8 and -9 were used for western blotting. Ctl: control untreated cells.*

Results from Figure 1A and B demonstrate that chlorogenic acid induced cytotoxicity (*i.e.* reduced the percentage of viable cells). In order to confirm whether chlorogenic acid induces cell death through the induction of apoptosis of U937 cells or not, we used DAPI staining for examining the apoptosis and TUNEL assays to show the presence of DNA fragmentation. Results from DAPI and TUNEL assay also showed that chlorogenic acid induced apoptosis (DNA fragmentation) in U937 cells.

It is well-documented that apoptosis can be divided into caspase-dependent and -independent and mitochondria-dependent and -independent signal pathways (6, 7). Our results showed that chlorogenic acid induced apoptosis through the activation of caspase-3 which was measured by using substrate of caspase-3. We also used the caspase-3 inhibitor, z-VAD-FMK, which significantly reduced the caspase-3 activation and increased the percentage of viable cells upon treatment with chlorogenic acid (data not shown).

It has also been reported that agents which induce apoptosis can be divided into mitochondria-dependent and -independent pathways (7). If an agent induced apoptosis through Fas-FasL then activated caspase-8, followed by caspase-3 then led to apoptosis, then this is called mitochondria-independent (caspase-dependent) pathway. If an agent led to cytochrome *c* release and promoted caspase-9 activation then led to apoptosis or AIF and Endo-G release, to cause apoptosis, then this is called mitochondria-dependent pathway (29, 30). Here, we also used flow cytometry to assay the levels of mitochondia membrane potential and results indicated that chlorogenic acid decreases the levels of $\Delta\Psi m$ in U937 cells (Figure 4B). This indicated that chlorogenic acid induced apoptosis in U937 cells through a mitochondria-dependent pathway.

It is well-known that ROS play an important physiological role, such as they can act as secondary messengers to promote or suppress the expression of a number of genes and/or signal transduction pathways (31, 32). It was reported that maintenance of the homeostasis of ROS is critical in cell signaling and in the regulation of cell death (31). It was also reported that tumor cells have higher levels of ROS than their normal counterparts; tumor cells are more sensitive to the additional oxidative stress generated by anticancer agents (33). Here, we also used flow cytometric assays for measuring the ROS production in U937 cells, after exposure to chlorogenic acid, and the results indicated that chlorogenic acid promoted ROS production in U937 cells (Figure 4). This is in agreement with another report demonstrating that chlorogenic acid induced apoptosis *via* ROS production in cancer cells (18). Thus, we also suggest that chlorogenic acid induced apoptosis of U937 cells through the ROS production.

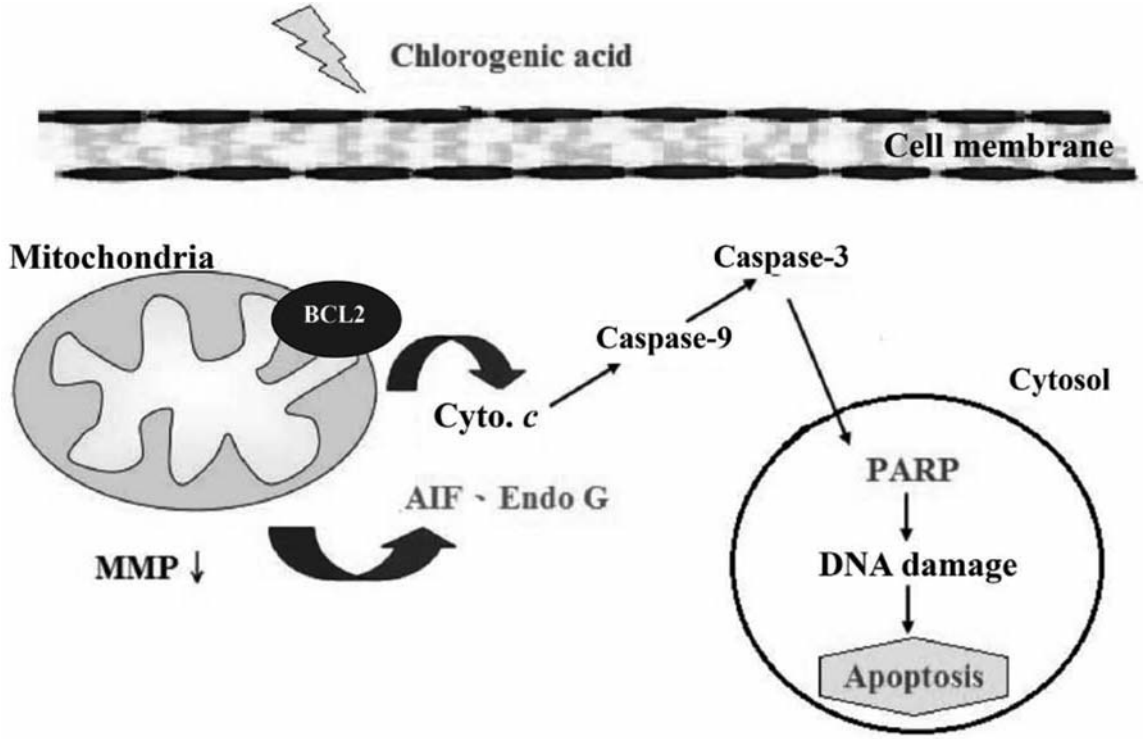

Figure 7. *Proposed model for chlorogenic acid-triggered apoptosis of human leukemia U937 cells. BCL-2, B-cell lymphoma 2; Cyto c, cytochrome c; PARP, Poly (ADP-ribose) polymerase; AIF, apoptosis-inducing factor; Endo G, Endonuclease G.*

In conclusion, our study suggests that chlorogenic acid-induced cytotoxic effects occur through induction of apoptosis by the disruption of the mitochondrial membrane potential (reduction of $\Delta\Psi m$), ROS production, activation of caspase-3, -7, -8 and -9 induction of apoptosis, as summarized in Figure 7.

Conflicts of Interest

None of the Authors have any conflict of interest to declare.

Acknowledgements

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