Abstract. Chlorogenic acid exists widely in edible and
toxic 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
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 (ΔΨm), 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 ΔΨm and by increasing the activation of
caspase-3 pathways in human leukemia U937 cells in vitro.

Development of drug resistance in tumor cells and side-
effects in patients have led to limitations to current
therapy 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-cm² culture flasks and were grown in RPMI-1640 supplemented with 10% FBS, penicillin-streptomycin, trypsin-EDTA and 4,6-diamidino-2-phenylindole dihydrochloride (DAPI), as described previously (21, 22).

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determination of cell viability and morphology. U937 cells were plated at a density of 5×10⁵ 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% CO₂ and 95% air for 48 h. Cells from each treatment were harvested and re-suspended in 500 μl of 10 μM (DCFH-DA; 2,7-dichlorodihydrofluorescein diacetate) for ROS and in 500 μl of 1 μM dihexyloxacarbocyanine iodide (DiOC₆) for ΔΨm. 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 1×10⁷ 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 washed 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...
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 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.
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 activity 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 ΔΨm 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 ΔΨm 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 ΔΨm (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.
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).
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 mitochondria 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.

![Western Blot Analysis](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.)
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 ΔΨm), 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.

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References


