**Houttuynia cordata** Thunb extract induces apoptosis through mitochondrial-dependent pathway in HT-29 human colon adenocarcinoma cells

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**Abstract.** The *Houttuynia cordata* Thunb (HCT) extract has been used as a traditional Chinese herb medicine and as well as an effective drug for treating allergic inflammation for thousands of years. In this study, we investigated the anti-cancer activity of HCT and its molecular mechanisms in the human colon adenocarcinoma cell line HT-29. HCT inhibited HT-29 cell viability in a dose- and time-dependent manner by MTT assay. Treatment with 450 μg/ml of HCT for 48 and 72 h led to DNA damage and apoptosis by DAPI staining and comet assay. HCT increased reactive oxygen species production and decreased the levels of mitochondria membrane potential (MMP) in HT-29 cells by flow cytometry analysis. HCT released the release of cytochrome c, Apaf-1, pro-caspase-9 and AIF from mitochondria via a decrease of the MMP. The decrease of MMP was then associated with a decrease in the ratio of Bax/Bcl-2 and activation of caspase-9 and -3 by Western blotting and caspase activity assay. Caspase-9 and -3 inhibitors almost completely suppressed HCT-induced caspase-9 and -3 activities. Our results demonstrated that the HCT-induced apoptosis in human colon adeno-carcinoma cell line HT-29 might be related to a mitochondrial-dependent pathway.

**Introduction**

In Taiwan, ~15.03 persons per 100,000 people died per year from colon cancer, according to the reports of the ‘People Health Bureau of Taiwan’ (1). In clinical practice, surgery, radiotherapy and chemotherapy are used for treating colon cancer patients (2). However, the current strategies for treatment of human colon cancer are limited and the results are not yet satisfactory. Induction of apoptosis in cancer cells has been considered as an important strategy in cancer treatment (3). Many anti-cancer drugs can inhibit tumor cell growth by apoptosis induction (4,5).

Apoptosis is a normal component of the development and physical condition of cellular organisms. Many studies have focused on selectively killing cancer cells through the induction of apoptosis (6,7). Apoptosis is a kind of cell death that causes specific morphological modification such as cell membrane blebbing, chromatin condensation, caspases activation and DNA fragmentation that are considered landmarks of the apoptotic process (8-10). Present studies demonstrated that mitochondria are important in regulation of apoptosis (11,12). Mitochondrial changes include decrease of mitochondrial membrane potential (MMP), increase of reactive oxygen species (ROS) production, release of cytochrome c, Apaf-1 and pro-caspase-9 proteins from mitochondria to cytosol and then activation of caspases (11,13,14). The activation of effector caspases (caspase-3, -6 and -7) by initiator caspases (caspase-2, -8, -9 and -10) are responsible for the cleavage of cellular substrates including lamin and poly (ADP-ribose) polymerase (PARP) (15,16). Cleavage of those substrates degrades the chromosomes into nucleosomal fragments during apoptosis (17).
Thunb is a perennial herb that is native to Southeast Asia (18) and it has a thin stalk and heart-like leaf. It is called E-Sung-Cho in China and it is known to be an effective drug for treating allergic inflammation (19). Previous studies concluded that the HCT is effective for treating anaphylaxis, cancer and viral infection (20-22). Recently, many studies also demonstrated anti-leukemic activity (21), anti-anaphylaxis, cancer and viral infection (20-22). Recently, many studies concluded that the HCT is effective for treating allergic inflammation (19).

Preparation of HCT. Hottuynia cordata Thunb 50% ethanol extracts of PR (yield: 6.73% of dry wt.) were obtained by 48 h maceration at room temperature. The ethanol extract was filtered through a 0.45-μm filter (Osmonis, Minnetonka, MN, USA), lyophilized and kept at 4˚C. The dried extract was resolubilized in PBS before use.

Chemicals and reagents. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI); DAPI; low-melting agarose (Sigma, St. Louis, MO); RPMI-1640, fetal bovine serum (FBS), trypsin-EDTA and penicillin/streptomycin (Gibco BRL). Proteinase K was purchased from Roche Diagnostics (GmbH, Mannheim, Germany). The Bio-100TM DNA Ladder marker was obtained from PROtech Technology Enterprise Co. (Taipei, Taiwan). All other chemicals used were of analytical grade.

Cell culture. Human colon adenocarcinoma cell line HT-29 was obtained from the American Type Culture Collection. Cells were maintained in RPMI-1640 containing 100 ml/l FBS with 100,000 U/l penicillin and 100 mg/l streptomycin (24).

MTT assay. HT-29 cells were plated onto 96-well plates and exposure to HCT as detailed in respective experiments for 24 and 48 h. MTT was added to each well then incubated for an additional 4 h in dark at 37˚C. The medium was then aspirated from the wells and the blue formazan product was dissolved in 100 μl of DMSO. The plates were analyzed at O.D. 570 nm using a spectrophotometric plate reader (Bio-Rad, Tokyo, Japan). Each data point was replicated in triplicate. Percentage of cell viability was calculated as (O.D. of drug-treated sample/O.D. of non-treated sample) x100% (24).

DAPI staining. After HCT treatment, cells were fixed in 4% paraformaldehyde for 30 min and incubated with 1 μg/ml of DAPI staining solution for 30 min in the dark. The apoptotic cells were observed through fluorescence microscopy (Zeiss, Oberküchen, Germany) (25).

Comet assay. Each slide of 10,000 cells was mixed with 150 μl 0.75% low-melting agarose (Sigma type VII, USA) held at 37˚C. The agarose was spread into single layers on ordinary, clear-glass slides that had been pre-treated with a small amount of agarose and air-dried. After solidifying on a chilled plate, the slides were transferred to the same lysis buffer and held at room temperature for 4 h, when appropriate in the presence of DAPI as above (26).

ROS production assay. ROS was measured after staining with DCFH-DA dye in HT-29 cells. Cells were collected and washed with PBS. One ml of PBS containing 20 μM DCFH-DA was added and the cells were incubated for 30 min at 37˚C. The fluorescence emission from DCF was analyzed by flow cytometry (Becton-Dickinson, San Jose, CA) (27).

MMP assay. Changes of MMP were monitored after staining with DiOC6. HT-29 cells were collected, trypsinized and washed in PBS, then they were stained with DiOC6 (5 μg/ml) for 30 min at 37˚C. The percentage of green fluorescence was estimated by flow cytometry (28).

Western blotting. Total cell lysates were prepared, as described previously (29,30). Assay kits for cytosolic proteins (Calbiochem, Germany) were used to assess the release of cytochrome c, Apaf-1, AIF and pro-caspase-9 from mitochondria to cytosol and the purity of the fractions estimated (31). The 30 μg proteins were resolved on SDS-PAGE and transferred onto a polyvinylidene fluoride membrane (PVDF; Millipore). After blocking, the blots were incubated with an appropriate dilution of specific monoclonal antibodies for cytochrome c, Apaf-1, pro-caspase-9, AIF, Bax, Bcl-2, and xIAP (Santa Cruz Biotechnology, USA) for 12 h. Blots were washed three times and then incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology). The specific protein was detected by using enhanced chemiluminescence kits (Amersham, ECL kits) (30).

Caspase activity assay. HT-29 cells were collected in lysis buffer (50 mM Tris-HCl, 1 mM EDTA, 10 mM EGTA, 10 mM digitonin and 2 mM DTT) on ice for 10 min. The lysates were centrifuged at 15,000 x g at 4˚C for 10 min. Cell lysates (50 μg protein) were incubated with caspase-3, -9 and -8-specific substrates (Ac-DEVD-pNA, Ac-LEHD-pNA and Ac-IETD-pNA) with reaction buffer in a 96-well plate at 37°C for 1 h.
The caspase activity was determined by measuring OD 405 of the released pNA (31).

Statistical analysis. Student’s t-test was used to analyze differences between HCT-treated and control groups. *P<0.05, **P<0.01, ***P<0.001.

Results

HCT inhibits cell viability of HT-29 cells. Cells were treated with HCT at concentrations of 0, 250, 500 and 1000 μg/ml. Cell viability was determined by MTT assay 24, 48 and 72 h later. As shown in Fig. 1, HCT inhibited cell viability in HT-29 cells in a dose- and time-dependent manner. The concentration required for inhibiting growth by 50% (IC50) for 72 h on HT-29 cells was ~435 μg/ml.

HCT induces morphological changes and DNA damage in HT-29 cells. To verify whether HCT could inhibit cell viability through induction of apoptosis and DNA damage in HT-29 cells, we assessed the nuclear morphological change by DAPI staining and DNA damage by Comet assay (single cell gel electrophoresis). As shown in Fig. 2A and B, after 48 and 72 h incubation with 450 μg/ml of HCT, cells exhibited nuclear shrinkage and chromatin condensation. Less than 1% of the control cells showed evidence of DNA damage in the form of the typical tail formation. Cells exposed to HCT exhibited dose-dependent increase in DNA damage by Comet assay. Our results demonstrated that the HCT could induce morphological change and DNA damage in HT-29 cells.
HCT induces ROS production and loss of MMP in HT-29 cells. To verify whether HCT could cause cell apoptosis through induction of mitochondria stress in HT-29 cells, we measured the intracellular ROS level and MMP change. As shown in Fig. 3, HT-29 cells were treated with 450 μg/ml of HCT, cells showed significant decrease in MMP and increase level of ROS. Our results suggested that the mitochondria dysfunction may be involved in HCT-induced apoptosis and ROS may play a key role in this pathway.

HCT induces apoptosis by the activation of Caspase-9 and -3. We investigated the HCT-treated HT-29 cells for caspase-9, caspase-8 and caspase-3 activities by colorimetric enzymatic assay. As shown in Fig. 4A, B and C, both caspase-9 and -3 activities increased at 12 h after HCT treatment. Our results suggested that the HCT-induced apoptosis was mediated through the activation of caspase-9 and -3. To further verify the involvement of caspases-9 and caspases-3 in HCT-induced apoptosis of HT-29 cells, caspase-9 inhibitor (Z-LEHD-FMK) and caspase-3 inhibitor (Z-DEVE-FMK) were pretreated. Our results demonstrated that Caspase-9 inhibitor (Z-LEHD-FMK) and caspase-3 inhibitor (Z-DEVE-FMK) almost completely suppressed HCT-induced caspase-9 and -3 activity and they also increased the viable HT-29 cells.

Effects of HCT on levels of apoptosis-associated proteins in HT-29 cells. To study the mechanisms of HCT induced apoptosis, we investigated the protein expression of cytochrome c, Apaf-1, AIF and pro-caspase-9 in cytosolic lysates and Bcl-2, xIAP, Bax protein expression by Western blot analysis. Fig. 5A shows the release of cytochrome c, Apaf-1, AIF and pro-caspase-9 from the mitochondria into the cytosol after treatment with HCT for 24 h. As shown in Fig. 5B, HCT decreased the levels of Bcl-2, xIAP and increased the levels of Bax. Our results suggested that the HCT-induced apoptotic response is mediated by a mitochondrial pathway.
Discussion

*Houttuynia cordata* Thunb (HCT) has been used as food and traditional Chinese herb medicine in South Asia including Taiwan for a very long time. The biologic activity of HCT includes anti-microbial, anti-viral, immunoo-regularatory, diuretic, anti-inflammatory and anti-leukemic effects (19-23,32). Although HCT was reported to be active against tumors, its anti-colon cancer effect has never been well investigated before. Our studies revealed that 450 μg/ml of HCT might have anti-colon adenocarcinoma activity (Fig. 1) and the activity was concentration- and time-dependent. Previous studies showed that the HCT contains quercetin 3-β-D-glucoside (isoucitrin) which has anti-HSV activity (33) and quercetin 3-D-galactoside (hyperin) which can inhibit SARS, HBV and herpes viruses (34). HCT also contains chlorogenic acid which has anti-pyretic, anti-HSV and anti-adenovirus effects (35). In this study, we demonstrated that the 50% ethanol extracts of HCT might have anti-colon adenocarcinoma activity. However, further fractionation of the 50% ethanol extracts in anti-colon adenocarcinoma assays would be required for identification of the bio-active components.

Previously, Chang et al demonstrated that HCT could inhibit five types of leukemia cells (L1210, U937, K562, Raji and P3HR1 cells) with IC₅₀ between 478 μg/ml and 662 μg/ml (21), but the molecular mechanisms of HCT-induced cell death remained unclear. Our results suggested that HCT might exert cytotoxicity by induction of apoptosis and then leads to cell death in HT-29 cells. Based on the results from DAPI staining, Comet assay (Fig. 2A and B) and caspase-3,-9 activity assay (Fig. 4B and C), we demonstrated the HCT might induce apoptosis through caspase-dependent response in HT-29 cells *in vitro*. Taken together, these findings provide important new possible molecular mechanisms for the anti-colon adenocarcinoma activities of HCT on HT-29 cells.

Apoptosis has two major pathways: the extrinsic (death receptor) pathway and the intrinsic (mitochondria) pathway (11,12). The extrinsic pathway involves Fas, TNF receptor and caspase-8 which drives its activation and then activates caspase-3 (36). The intrinsic pathway involves the signals to mitochondria, which lead to the release of cytochrome c, Apaf-1, AIF and pro-caspase-9, and then combine with Apaf-1 and pro-caspase-9 to form the apoptosome (11,13,14). Park et al demonstrated that HCT inhibited nitric oxide (NO) production in a dose-dependent manner, but minimally inhibited TNF-α secretion at 0.0625 and 0.125 mg/ml on macrophage-like cell line (RAW 264.7 cells) (37).

In our results, we found that the HCT does not affect the protein levels of Fas, FasL (data not shown) and caspase-8 activity (Fig. 4A) in HT-29 cells. We found that the loss of MOMP, the increase of ROS production and the alterations of mitochondrial proteins such as cytochrome c, Apaf-1, AIF and pro-caspase-9 in HCT-treated HT-29 cells may suggest that the mitochondria was the major target of HCT (Fig. 5). We also found that by adding caspase-9 and caspase-3 inhibitors, we could prevent HCT-treated HT-29 cells from apoptosis (Fig. 4B and C). We concluded that the HCT-induced apoptotic cell death is mediated through the activation of caspase-cascades and that it is mitochondria-dependent.

Our novel study demonstrates the involvement of mitochondrion-dependent pathways in HCT-induced apoptosis in HT-29 cells.

In conclusion, our results demonstrated that 450 μg/ml of HCT may induce apoptosis in human colon adenocarcinoma HT-29 cells through a mitochondria-dependent pathway.

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References


