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Cytotoxic and Apoptotic Activities of *Tussilago farfara* Extract in HT-29 Human Colon Cancer Cells

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Abstract The flower buds of *Tussilago farfara* (TF) have been traditionally used in oriental medicine for the treatment of bronchitis and asthma. In our study, the primary objective was to determine the mechanisms that are inherent to TF-induced cytotoxicity and apoptosis, using the methanolic extract of TF (TFM) in HT-29 human colon cancer cells. We found that TFM-induced induced cytotoxicity in HT-29 cells in a dose-dependent manner. This effect was verified via an MTT reduction assay, an lactate dehydrogenase (LDH) release assay, and a colony formation assay. Interestingly, we also detected apoptotic bodies on Hoechst staining, and attempted to determine whether TFM-induced apoptosis involved the caspase pathway using a caspase-3/7 activity assay. Overall, the results indicate that TFM contain chemotherapeutic agents and potential candidates use for against human colon cancer cells.

Key words: *Tussilago farfara*, oriental medicine, HT-29 cell, cytotoxicity, apoptosis

Introduction

In cases where injury is caused by an extrinsic stimulus, most normal cells repair immediately, or undergo apoptosis to maintain equilibrium. However, when the maintenance of cell proliferation and cell death is disrupted for whatever reason, these cells invade other tissues and organs (1). From a physical point of view, changes in resonance and homeostasis may induce a disease state (2).

Solid tumors in their multifarious forms are one of the principal causes of human death in the current era (3). Specifically, colon cancer is among the most prevalent and deadly cancers and its incidence increasing in Asian countries. Conventional treatments such as surgical resection, radiation therapy, and chemotherapy are not satisfactory, and the prevention of this disease at its inception is important (4). Cancer therapies and anticancer drugs are continuously being examined by researchers (5-9). Anticancer drugs are available for the treatment of this disease, but the majority of patients do not respond to these drugs and side effects remain problematic (10). Accordingly, it is advisable to explore a new approach for developing effective therapies against this disease. Unfortunately the current therapeutic modalities for advanced disease are limited.

Alternative options for patients with malignancies include herbal treatments that have been used for many years throughout the world (11-13). A host of natural products are shown to have pharmacological applications, and may have some potential in chemotherapeutic uses (14-16). Herbal medicines have been extensively tested because of their low toxicity and considerable medicinal value (17), and the use of alternative herbal medicines for cancer prevention and treatment has been increasing significantly (18).

The flower buds of *Tussilago farfara* (TF), also known as '*kwandong-hwa*' in Korea, have been used in traditional oriental medicine for the treatment of bronchitic and asthmatic conditions (19,20). TF has several known pharmacological activities, namely antimicrobial activity (21), inhibitory activity against nitric oxide synthase (22), and restraint of the platelet-activating factor receptor (23). Although, some of the biologic effects and pharmacological activities of its constituents have been clarified (24), TF's mode of action remains unclear, and the apoptotic mechanism associated with the anti-proliferation effect of TF has not, until now, been elucidated.

Therefore, the purpose of this study was to assess the cytotoxic and apoptotic activities of the methanolic extract from TF (TFM) in HT-29 cells, and subsequently, we have proposed the mechanisms responsible for its cell death activity.

Materials and Methods

Materials *Tussilago farfara* (TF) was obtained from Kumkang Pharm Co., Ltd., Masan, Korea. 3-(4,5-Dimethylthiazol-2-yl)-2,5-dipheny-tetrazolium bromide (MTT) was provided by Sigma Chemical Co. (St. Louis, MO, USA). A lactate dehydrogenase (LDH) release assay kit was purchased by Wako Pure Chemical Industries, Ltd. (Osaka, Japan). RPMI1640 medium, fetal bovine serum (FBS), and penicillin/streptomycin were obtained from Gibco BRL (Grand Island, NY, USA). All organic solvents and other chemicals were of analytical grade or complied with the requirements for cell culture experiments.

Preparation of methanolic extracts from TF Each 5 g of TF was extracted with 100 mL of methanol for 3 days at room temperature and filtered through a Whatman No. 1 filter paper (Advantec, Tokyo, Japan). The methanol was removed by evaporation *in vacuo*, and a dried methanol extract was obtained. The methanolic extract from TF was called TFM. The TFM was then dissolved in dimethyl sulfoxide (DMSO) at a concentration of 25 mg/mL for experiments.

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Cell culture and treatments HT-29 human colon cancer cells were obtained from Korean Cell Line Bank. The cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, penicillin (100 U/mL), streptomycin (100 μg/mL), and 2 mg/mL of $NaHCO_3$ in a humidified 37°C incubator gassed with 5% CO_2. TFM was dissolved in DMSO and added to the culture medium so that the final concentration of DMSO was less than 1%.

MTT reduction assay for cell viability Cell viability was measured with blue formazan that was metabolized from MTT by mitochondrial dehydrogenase, which are active only in live cells. HT-29 cells preincubated in 96-well plate at a density of 1×10^5 cells/mL for 24 hr in humidified atmosphere of 5% CO_2 at 37°C. Cells were pretreated with various concentrations of TFM. After incubation for 24 hr, MTT reagent (5 mg/mL) was added to each of the wells, and the plate was incubated for an additional 1 hr at 37°C. The media were then removed, and the intracellular formazan product was dissolved in 100 mL of DMSO. The absorbency of each well was then measured at 540 nm using the enzyme-linked immunosorbent assay (ELISA) reader (model 680; BioRad, Hercules, CA, USA), and the percentage viability was calculated.

LDH release assay Cytotoxicity was determined by measuring the release of LDH. HT-29 cells were pretreated with various concentrations of TFM for 24 hr, and the supernatant was used to assay LDH activity. The reaction was initiated mixing 50 μL of cell-free supernatant with potassium phosphate buffer containing nicotinamide adenine dinucleotide and sodium pyruvate in a final volume of 100 μL in a 96-well plate. A colorimetric assay was performed, according to which the amount of formazan salt is proportional to LDH activity in the sample. The intensity of red color formed in the assay and measured at 540 nm was proportional to LDH activity and to the number of damaged cells. Data were normalized to the activity of LDH released from vehicle-treated cells and expressed as percentage of the control (obtained in separate plating).

Colony formation assay HT-29 cells were seeded at 5×10^4 cells/mL in 24-well plates, incubated overnight, and treated with different concentrations of TFM for 24 hr. The cells were then diluted in new medium, replated at 1×10^3 cells/mL in 6-well plate, and cultured under normal growth conditions for 7 or 8 days at 37°C in a humidified atmosphere containing 5% CO_2 to form colonies. The colonies were fixed with 10% formaldehyde, stained with 0.01% crystal violet, and counted. Cell survival (mean values with 95% confidence intervals from triplicate determinations) was calculated by normalizing the survival of control cells as 100%.

Cell staining HT-29 cells were washed twice with phosphate-buffered saline (PBS) and then fixed in PBS containing 10% formaldehyde for 2 hr at room temperature. Fixed cells were washed with PBS, and stained with Hoechst 33342 (Sigma Chemical Co.) for 1 hr at room temperature. The cells were washed twice more with PBS and the Hoechst-stained nuclei were visualized by using a fluorescence microscope.

***In vitro* caspase-3/7 activity assay** Caspase-3/7 activity was measured with the AnaSpec homogeneous assay kit (AnaSpec Inc., San Jose, CA, USA) using a synthetic fluorometric substrate for caspase, AC-DEVD-AFC (25). Briefly, TFM-treated cells for different times were lysed in the supplied lysis buffer. The supernatants were collected and incubated with the 50 mL of homogeneous. Caspase-3/7 substrate solution was added to each well in 96-well plate. The 96-well plate was incubated at room temperature and assayed on a fluorescence spectrometer (excitation 360 nm, emission 535 nm). Data were expressed as fold increase in caspase activity of apoptotic cells over that of non-induced cells.

Western blot analysis Cells were lysed in 1× sodium dodecyl sulfate (SDS) sample buffer (62.5 mM Tris-HCl [pH 6.8], 2% SDS, 5% 2-mercaptoethanl, and 10% glycerol), and protein concentration of the lysates were measured with a Bio-Rad protein assay kit. Equal amounts of proteins were resolved by SDS-PAGE and transferred onto an immunoblot nitrocellulose transfer membrane (Scheicher & Schuell, Protran, Germany) for Western blotting. Membranes were probed with anti-human caspase-3 (Cell signaling, Danvers, MA, USA). Anti-rabbit IgG HRP (Amersham Pharmacia Biotech, Tokyo, Japan) was used as a secondary antibody. Protein loading was controlled by probing the membranes for β-actin protein. Western blots were developed using the enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech) according to the manufacture's instructions.

Fluorescence-activated cell sorter analysis Flow cytometric analysis of cellular DNA content was performed as described previously (26). Briefly, HT-29 cells were harvested and fixed with ice-cold 70% ethanol. The fixed cells were stained with 50 mg/mL of propidium iodide at room temperature in the dark for 30 min. Apoptotic cells (mean values with 95% confidence intervals from triplicate determinations) were measured using a FACS caliber flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

Statistical analysis All data are the means of 3 determinations and data was analyzed using the SPSS package for Windows (Version 11.5). The data were evaluated by one-way analysis of variance (ANOVA) followed by Scheffe's test. The differences were considered significant at $p<0.05$.

Results and Discussion

Cytotoxic activities of TFM on HT-29 human colon cancer cells To characterize the cytotoxicity that occurred in TFM-treated HT-29 human colon cancer cells, the cells were incubated with TFM, and morphological alterations were verified via a phase-contrast microscope. As shown in Fig. 1, after 24 hr of incubation with various concentrations of TFM, many of the cells showed cytoplasmic shrinkage, and either detached from each other or floated in the medium.

Next, we attempted to determine the cytotoxic effects of TFM via a MTT-dye reduction assay and a cytoplasmic LDH release assay. The HT-29 cells were treated for 24 hr with various concentrations of TFM. As shown in Fig. 2A,

TFM showed 20 to 60% reductions in cell viability compared to the control, and it also demonstrated a dose-dependent inhibition of cell growth. In particular, intensive cytotoxicity was induced in the HT-29 cells when they were treated with 250 μg/mL of TFM, with an IC_{50} value of 50 μg/mL.

Furthermore, we attempted to determine the cytotoxic effects of TFM via an LDH release assay, evaluating the extent of LDH leakage into the medium. We conducted this experiment in order to estimate the release of LDH after treatment with various concentrations of TFM for 24 hr against the HT-29 cells. As expected, TFM-induced cell death in a dose-dependent manner, as demonstrated by a 25 to 50% increase in LDH release in the TFM-treated HT-29 cells (Fig. 2B).

To clarify these results further, we examined the effects of TFM on cell viability by a colony forming assay (27). The inclusion of TFM in the culture medium was highly toxic to the HT-29 cells, and a TFM level of less than 50 mg/μL was required for 50% inhibition of colony formation (IC_{50}) (Fig. 2C). Taken together, these results clearly indicate that TFM can induce cytotoxic activity against HT-29 cells.

TFM-induced apoptosis in HT-29 human colon cancer cells To determine whether TFM's cytotoxic activity was due to apoptosis, the HT-29 cells were treated for 24 hr with various concentrations of TFM. HT-29 cells stained with Hoechst 33342 (10 μM) revealed marked chromatin condensation and apoptotic body formation when examined using a fluorescence microscope (Fig. 3), confirming that TFM-induced apoptosis in the HT-29 cells.

We determined that caspase-3 activity performs a central function in the pathway for apoptosis (28). Therefore, to verify whether caspase pathways were involved in TFM-induced apoptosis, caspase-3/7 activity was evaluated with a caspase-3/7 activation kit. Using a synthetic fluoro-metric substrate for the caspase, we determined a significant elevation of this enzyme's activity in HT-29 cells treated with TFM (Fig. 4A). Next, we conducted Western blot analysis to determine the protein level of the caspase-3. By treating the HT-29 cells with 10, 50, 100, and 250 μg/mL of TFM for 24 hr, we demonstrated cleavage of caspase-3 to the 19 kDa active form (Fig. 4B). These results indicate that TFM's anti-proliferation effect on HT-29 cells were attributable to apoptosis associated with caspase-dependent activation.

To examine the functions of apoptosis, we analyzed HT-29 cells that were similarly sensitized to TFM using flow cytometric assays. The apoptotic cells were distributed according to the cell cycle phase by showing sub-G1 DNA content. The amount of apoptotic cells that measured in the sub-G1 phase were approximately 5-fold greater for the

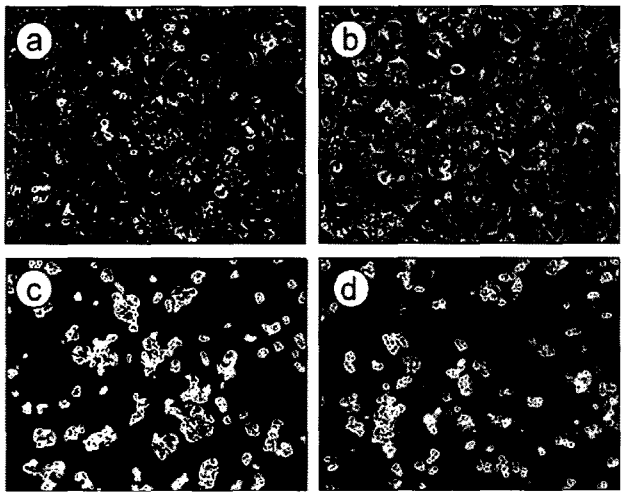

Fig. 1. TFM-induced cell death in HT-29 human colon cancer cells. The cells were exposed to various concentrations of TFM, and morphological changes were monitored for 24 hr (a, control; b, 50 μg/mL; c, 100 μg/mL; d, 250 μg/mL). Photographs were taken with a phases-contrast microscope at 200× magnification. The results are representative of at least 2 independent experiments.

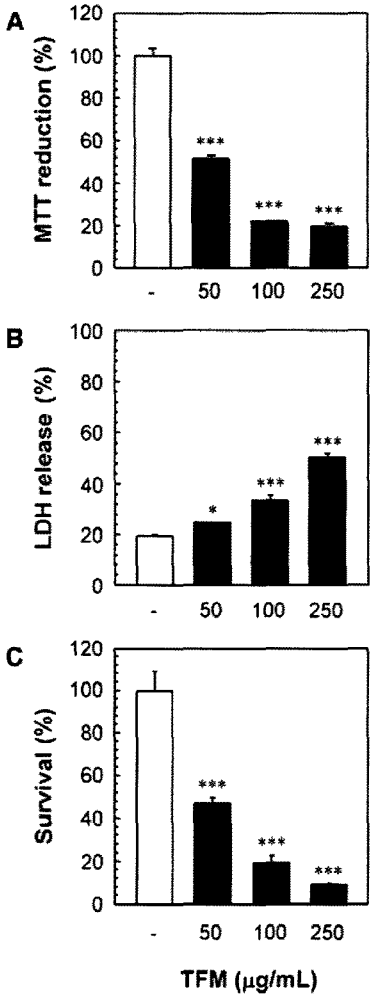

Fig. 2. Effect of TFM on cell viability and cytotoxicity of HT-29 cells. Cell viability and cytotoxicity were measured with the MTT reduction assay (A), and LDH release assay (B), and colony formation assay (C). The MTT assay, the MTT reduction rate was calculated by setting each of control survivals. As the results of LDH release assay, data were normalized to the activity of LDH released form vehicle-treated cells (100%) and expressed as percentage of the control (obtained from separate plating). Data (means±SD of 3 determinations) are representative of at least 3 independent experiments. Significant vs. control of untreated cells (*$p<0.05$; ***$p<0.001$).

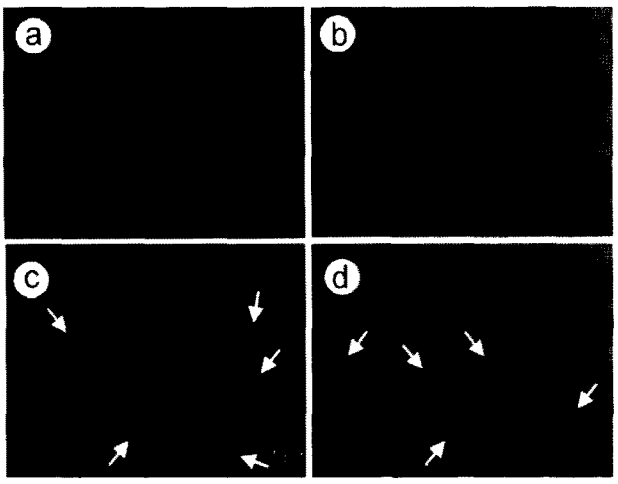

Fig. 3. TFM-induced apoptosis in HT-29 cells. Cells were collected for the following different experiment for apoptosis induction morphological features of HT-29 cells treated with various concentration of TFM (a, control; b, 50 μg/mL; c, 100 μg/mL; d, 250 μg/mL). The arrow indicates apoptotic cell. The results are representative of at least 2 independent experiments.

250 μg/mL TFM treatment compared with the control. These cell cycle analyses also clarified that TFM lead to a depletion of cells in the G1-phase and a concomitant accumulation of cells in the G2/M phase. G2/M arrest was also accompanied by an increase in the sub-G1 region of cells, which is typical for late stages of apoptosis (Fig. 4C).

In conclusion, our results clearly demonstrate that TFM significantly induces cytotoxicity and apoptosis in HT-29 human colon cancer cells. In addition, TFM activated caspase-3, resulting in an increase of the sub-G1 phase in cell cycle. These results show that TFM may exert anticancer activity by the induction of apoptosis via a caspase-dependent pathway. Therefore, TFM may constitute a potential candidate as a novel therapeutic agent in the field of anticancer drug discovery.

Acknowledgments

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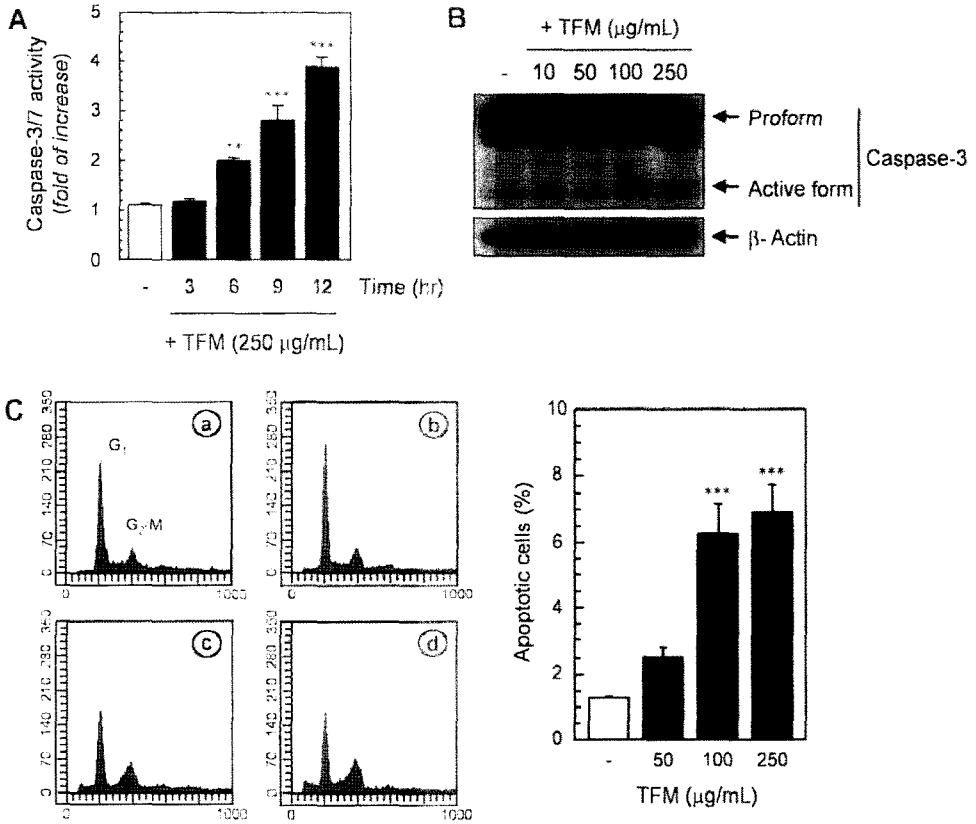

Fig. 4. Apoptosis effect of TFM in HT-29 cells. (A) The cells were treated with of without 250 μg/mL of TFM for different times. The caspase-3/7 activity of cell extracts was measured by the fluorescence assay. (B) Effects of TFM on caspase-3 activation. Caspase-3 activation was analyzed by Western blotting. (C) Flow cytometric analysis of apoptotic HT-29 cells at the indicated concentration of TFM (a, control; b, 50 μg/mL; c, 100 μg/mL; d, 250 μg/mL), and the cells were incubated for 48 hr. Data (means±SD of 3 determinations) are representative of at least 3 independent experiments. Significant vs. control of untreated cells (**$p<0.01$; ***$p<0.001$).

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