Combined Treatment with *Coptidis Rhizoma* Extract and Arsenic Trioxide Enhanced Apoptosis through Diverse Pathways in H157 Cells

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Coptidis rhizoma (huanglian) is an herb that is widely used in traditional Chinese medicine that has recently been shown to possess anticancer activity. However, the molecular mechanism underlying the anticancer effects of this herb is poorly understood. In this study, we investigated the anticancer activity of a combination of CR extract and arsenic trioxide, as well as the apoptotic pathway associated with its mechanism of action in human lung cancer H157 cells. Combined treatment of H157 cells with CR extract and arsenic trioxide resulted in significant apoptotic death. In addition, combined treatment with CR extract and arsenic trioxide acted in concert to induce a loss of mitochondrial membrane potential ($\Delta\Psi$), the release of cytochrome c from mitochondria, and an increase in the expression of pro-apoptotic p53 and Bax protein, which resulted in activation of caspases and apoptosis. CR extract combined with arsenic trioxide also increased the lipid peroxidation, mRNA expression of DR4 and DR5 and caspase-8 activity. These data indicate that combined treatment with CR extract and arsenic trioxide enhanced apoptotic cell death in H157 cells through diverse pathways, including mitochondrial dysfunction and death receptors, particularly DR4 and DR5. Thus, this treatment may be an effective from of chemotherapy.

Key words : *coptidis rhizoma* (huanglian) (CR) extract, arsenic trioxide, combined treatment, apoptosis, diverse pathways, H157 cells

Introduction

The use of herbal medicine as an alternative cancer therapy has recently attracted a great deal of attention due to its low toxicity to normal cells and decreased resistance. In addition, many studies have demonstrated the anti-tumor effects of oriental herbal medicines in various cancer cells[1,4]. However, despite their broad use, there are few scientific data available regarding the safety and efficacy of herbal medicines used for cancer therapy.

Coptidis rhizoma (CR) is an herb that has been widely used in Eastern Asia including China, Japan, and Korea as an antimicrobial agent for the treatment of dysentery, gastroenteritis and other inflammatory conditions, such as pneumonia and infection of the head and face for several thousand years[5-8]. Recently, it has been reported that the

extract of CR possesses topoisomerase I inhibiting activity[9].

However, the role of CR as an anticancer agent has not yet been defined. Demonstration of the anticancer effect s of CR *in vitro* and identification of novel targets would provide a rationale for clinical development of this agent for use in cancer therapy.

Arsenic trioxide (As_2O_3) has been adopted from traditional Chinese medicine and is highly effective in the treatment of patients suffering from acute promyelocytic leukemia (APL) refractory to all trans -retinoic acid. Accordingly, As_2O_3 has become an extremely important component of the clinical management of leukemia in such patients[10-13]. Several studies have shown that As_2O_3 also exhibits potent growth inhibitory effects in several other cell lines of diverse malignant phenotypes, including cervical cancer, lung cancer, glioma, prostate cancer and breast cancer cells[13-15]. However, because solid tumors developed resistance to arsenic trioxide, it has not been widely used in the treatment of solid tumors. Despite the well-documented clinical efficacy of arsenic in leukemia therapy, the precise mechanisms that regulate the arsenic-dependent induction of apoptosis in

neoplastic cells have not yet been elucidated. Nevertheless, because of the variety of cellular response approaches available to arsenic trioxide, it has been suggested that a combination of arsenic trioxide and other chemotherapeutic agents may result in cytotoxic synergy.

The combined use of two or more chemotherapy agents is often advantageous because it may permit lowering of drug dosages and a consequential decrease in toxicity, thereby reducing the opportunity for the development of drug resistance by cancer cells and providing the potential for synergistic effects between drugs that act with different mechanisms. Thus, combination of As_2O_3 with other drugs that activate additional apoptotic signals or inhibit survival signals may provide a rational molecular basis for novel chemotherapeutic strategies.

In the present study, we investigated the antitumor activity of treatment with a combination of CR extract and arsenic trioxide against the human lung cancer cell line, H157. We demonstrated that treatment with this combination of CR extract and arsenic trioxide significantly increased the apoptotic response of H157 cells.

Materials & Methods

1. Preparation of herbal extracts

The water extract of *Coptidis rhizoma*(CR) was prepared as follows under optimal water extraction conditions. Briefly, CR was boiled in water and then filtered, after which the aqueous phase was concentrated using a rotary vacuum evaporator and then lyophilized. The stock solution of the CR extract was then prepared by dissolving the CR extract in DMSO (10 mg/ml) by vigorous vortexing for 2 min followed by sonication. After centrifugation (2000 × g, 10 min), the supernatant was collected and stored at –20℃ until use. The stock solution was then diluted in basal medium at the concentrations indicated for the experiments.

2. Cell culture and reagents

The human lung cancer cell line, H157, was obtained from the American Type Culture Collection (Rockville, MD) and maintained in RPMI-1640 medium (Gibco, Grand Island, NY) supplemented with heat-inactivated fetal bovine serum (Gibco), penicillin (100 U/ml) and streptomycin (100 $\mu g/m\ell$) under 5% CO_2 in a humidified incubator at 37℃. Arsenic trioxide (As_3O_2), propidium iodide (PI), 3-[4, 5-dimethylthiazol-2-yl]-2, 5-dephenyl tetrazolium bromide (MTT), human IgG and TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) were purchased from Sigma (St. Louis, MO, USA). JC-1 and DCF-DA were purchased from Molecular Probes (Eugene, OR). Antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). DR4: Fc and DR5: Fc was obtained from Alexis (Switzerland).

3. Assessment of cell viability

Cell viability was measured by an MTT colorimetric assay[16]. Briefly, cells were plated in 48-well culture plates at a density of 5× 10^4cells/well and allowed to adhere at 37℃ for 12 h. The various doses of CR extract or arsenic trioxide were then added, after which the cells were incubated for an additional 48 h. The cell viability was then measured using MTT. The ability of cells to form formazan crystals via active mitochondrial respiration was determined using a Microplate reader (Titertek Multiskan, Flow Laboratories, North Ryde, Austria) after dissolving the crystals in DMSO. The results were expressed as a percentage of control. All experiments were repeated at least three times, and the data were expressed as the means ± SE.

To evaluate the death receptor-related signal pathway, H157 cells were plated at a density of 2×10^4cells per well in 48-well plates and then incubated for 12 h. The cells were then treated with drug-free media (control) or media containing CR extract (50 $\mu g/m\ell$), arsenic trioxide (2.5 μM), TRAIL (100 ng/ $m\ell$), DR4: Fc (2 $\mu g/m\ell$), DR5: Fc (0.5 $\mu g/m\ell$) or control human IgG (1 $\mu g/m\ell$) for 48 h. Photographs of the culture plates were taken using a phase microscope.

4. Apoptosis assay

To assess the apoptotic cell death, morphological changes in the chromatin structure were detected by DAPI (4, 6-diamidine-2, phenylindole dihydrochloride). Briefly, the cells were washed with PBS and then fixed with 4% paraformaldehyde solution for 15 min at room temperature. The cells were then washed with PBS and stained with 1 $\mu g/$ $m\ell$ DAPI in the dark for 5 min. Finally, the nuclear regions of the cells were observed under a fluorescent microscope using a 330-380 nm excitation and a 420 nm emission filter. Flow cytometric analysis was used to measure the cellular DNA content. Briefly, the cells (5 x10^5)were seeded in six-well plates and then allowed to attach overnight. The cells were then treated with CR extract or arsenic trioxide alone or in combination for 48 h. Next, the cells were harvested by trypsin treatment, washed with cold phosphate-buffered saline (PBS, pH7.4) and then stained with PI solution (50 $\mu g/m\ell$ of propidium iodide, 100 $\mu g/m\ell$ RNase, and 0.1% Triton X-100 in PBS). The samples were then analyzed for DNA histograms and cell cycle phase distribution by flow cytometry

(FACSCalibur, BD Bioscience). Data from 10,000 cells per sample were collected and analyzed using the Cell Quest™ software (Becton Dickinson), and than cell death was determined by evaluating the percentage of events accumulated in the sub G_0/G_1 phase position.

5. Measurement of the mitochondria membrane potential (MMP)

The mitochondrial membrane potential of intact cells was measured by flow cytometry using a JC-1 lipophilic cationic probe (J-aggregate formation). In healthy H157 cells, the intact membrane allows the lipophilic dye, JC-1, to enter the mitochondria, where it accumulates and forms aggregates, producing an intense orange signal. In apoptotic cells in which the mitochondrial membrane potential has collapsed, the monomeric JC-1 remains in the cytosol and appears green. Thirty min before the cells were harvested, JC-1 was added directly to the culture medium to a final concentration of 30 nM, after which the cells were analyzed using a FACSCalibur instrument (BD Bioscience) equipped with the CellQuest Pro software. The apoptotic cells stained with JC-1 are represented in the lower right quadrant of the dot plot.

6. Western blot analysis

Cytosolic protein extracts were prepared as previously described[16]. Briefly, cells were collected by centrifugation at 300 x g for 5 min at 4℃ and then washed with ice-cold PBS. The cell pellet was then resuspended in 500 $\mu\ell$ of lysis buffer (20 mM Hepes- KOH, pH 7.5, 210 mM sucrose, 70 mM mannitol; 1.5 mM MgCl₂, 10 m MKCl₂, 10 $\mu g/m\ell$ leupeptin, and 10 µM digitonin). After 10 min of incubation at 25℃, the sample was spun at 14,000 x g for 15 min, and the supernatant containing cytosolic proteins was then stored at -70℃ until analysis by polyacrylamide gel electrophoresis (SDS-PAGE).

The protein extract was subjected to standard SDS-PAGE, transferred onto polyvinylidene difluoride membranes (Millipore), and then probed with appropriate antibodies as described in the figure legends. The bound primary antibody was detected using appropriate horseradish peroxidase-conjugated secondary antibody, and the protein was visualized using an enhanced chemiluminescence detection kit. Western blot an alysis was conducted using primary antibodies against cytochrome c, VDAC, Bax and p 53 (Santa Cruz Biotechnology) under the optimal dilutions. In addition, β-actin was used as an internal control to confirm equal loading.

7. Detection of cytochrome c release

Cytochrome c released from mitochondria was determined by western blot analysis. The cytosolic and mitochondrial fractions were then prepared as below, but the fractionation buffer contained 250 mM sucrose, 10 mM KCl, 1.5 mM MgCl₂, 1 mM dithiothreitol, 1 mM EDTA, 1 mM EGTA, 20 mM HEPES, pH 7.0, and protease inhibitors (1 mM sodium orthovanadate, 10 ㎎/㎖ leupeptin, 10 ㎎/㎖ aprotinin, and 1 mM phenylmethylsulfonyl fluoride). The mitochondrial pellet was resuspended in the same buffer, sonicated, and centrifuged to obtain the soluble fraction. Equal amounts of protein were then separated on a 15% SDS polyacrylamide gel and transblotted onto polyvinylidene difluoride-nitrocellulose filters. Next, the specific cytochrome c band was detected using anti-cytochrome c antibody (1:500) followed by enhanced chemiluminescence-based detection.

8. Measurement of intracellular ROS production

To monitor the intracellular ROS, we utilized the cell-permeable probes, DCFH-DA. Nonfluorent DCFH-DA hydrolyzed to DCFH inside of cells yields highly fluorescent DCF in the presence of intracellular hydrogen peroxide and related peroxides. Therefore, the DCF fluorescence intensity is proportional to the amount of hydrogen peroxide formed intracellulary. At the end of treatment, cells were washed once with PBS, and then incubated in growth media containing 10 µM DCF-DA for 30 min at 37℃. The cells were then washed once with PBS and suspended in cold PBS, after which the fluorescence was monitored by flow cytometry (FACSCalibur, BD Bioscience). As a positive control, cells were treated with CR extract and arsenic trioxide alone or combined and then processed for ROS detection.

9. Analysis of lipid peroxidation (MDA-TBA assay)

A lipid peroxidation assay was conducted using the method described by Ohkawa et al.,[17], with the modifications reported by Fordetal[18]. Briefly, 50 $\mu\ell$ of PBS resuspension of harvested cells (1-5 × 10⁶cells) were added 50 $\mu\ell$ of 3.2% SDS in PBS, and incubated for 10 min at room temperature.

Following incubation, 150 $\mu\ell$ of 20% acetic acid (pH 3.5) and 150 $\mu\ell$ of 0.8% TBA (thiobarbituricetcid)oin 0.05 N NaOH were added and the mixture was then boiled for 1 h. Next, the mixture was cooled and extracted with 1 ml of a netutanol-pyoinine mixture (1:3, v/v) to avoid turbidity. The upper layer (approximately 240 $\mu\ell$) of each sample was aspirated, after which the absorbance was measured at 532 nm.

The concentrations of 2-TBA were determined using the extinction coefficient of $1.56 \times 10^5 M^{-1}$ cm^{-1} and the results were expressed as mol of MDA (malondialdehyde) per mg per

protein. The level of protein was determined using the method described by Lowry et al.,[19], with bovine serum albumin as a standard.

10. Caspase activity assay

To measure the activity of caspase activity in H157 cells, a fluorogenic assay was used according to the manufacturer's protocols. Briefly, cells were treated with CR extract or arsenic trioxide alone or combined under the indicated conditions. The cells were then washed once in cold PBS and lysed in buffer containing 50 mM HEPES (pH 7.4), 100 mM NaCI, 0.5% CHAPS (pH 7.4), 1 mM DTT, and 0.1 mM EDTA. The cell lysate was then incubated with peptide substrate in assay buffer for 2 h at 37℃. Cleavage of fluorogenic substrates, including 100 µMsuc-DEVD-uMC, by caspase-3 was measured at an excitation of 380 nm and an emission of 460 nm. Cleavage of the fluorogenic substrate, Z-IETD-AFC (Calbiochem), by caspase-8 was measured using a spectrofluorometer (Jasco FR-777) at 400/505 nm. The results are reported as the percent change in activity as compared to the untreated control.

11. RT-PCR analysis

Total RNA was isolated from H157 cells using TRIzol[TM] Reagent (Gibco BRL, USA) according to the manufacturer's instructions. The cDNA was produced using oligo d (T) primers and reverse transcriptase (Promega, USA). The specificity of all primers was checked by sequencing of the PCR products. To ensure that the PCR signals detected were not caused by amplification of genomic DNA, control RT-PCR experiments were performed in which cDNA was synthesized without reverse trancriptase. The PCR consisted of 30 cycles of denaturation at 95℃ for 30 s, primer annealing at 60℃ for 1 min and elongation at 72℃ for 1 min using the following primers specific for human DR4 (5'-ATG GCG AGT GGG ACA GAG GCA-3', 5'-ATT ATG TCC ATT GCC TGA TTC-3') and human DR5 (5'-ATG GAG TCT GCT CTG ATC ACC CA-3', 5'-TGA GAG AGA ACA GGG AGA G-3'). In addition, primers specific for human β-actin (5'-GTG GGG CGC CCC AGG CAC CA-3', 5'-CTC CTT AAT GTC ACG CAC GAT TTC-3') were used as an internal standard. All experiments were repeated at least three times.

12. Statistical analysis

The data given in the text are expressed as the means ± S.D. Statistical comparisons among three or more groups were conducted using one-way analysis of variance (ANOVA). A difference was considered to be significant at *p< 0.05, or **p<0.01.

Results

1. Combined treatment with *Coptidis rhizoma* (CR) extract and arsenic trioxide (As$_2$O$_3$) enhanced cell death in H157 cells

To evaluate the effects of CR extract administered in combination with arsenic trioxide on cell viability, we investigated the cytotoxic effects of the combined treatment in H157 cells. Initial screening was conducted using an MTT assay to evaluate the effects of relative exposure to various concentrations of CR extract for 48 h. These experiments were conducted to identify a concentration of CR that produced modest cytotoxicity for subsequent testing in combination with arsenic trioxide. As shown in Fig. 1A, CR extractinhibited cell viability in a dose dependent manner. We next evaluated the cytotoxicity of CR extract in combination with arsenic trioxide. We noticed that a dose of CR extract as low as 50 µg/㎖ caused few cytotoxic effects (20%), but that significantly higher levels of cytotoxicity (40%) were observed when this dose of CR was combined with 2.5 mM arsenic trioxide(Fig. 1B). Hence, the effects of treatment with 50 µg/㎖ CR extract and 2.5 µM arsenic trioxide in H157 cells were evaluated. We next examined the effects of the combined treatment of CR extract and arsenic trioxide on H157 cells for various lengths of time (Fig. 1C). Combined treatment with CR extract and arsenic trioxide showed enhanced cytotoxicity in a time-dependent manner. Based on these findings, a combination of 50 µg/ml and 2.5 µM of CR extract and arsenic trioxide, respectively, was used in the following experiments.

2. Combined treatment with CR extract and arsenic trioxide induced apoptosis in H157 cells

To determine if the cell death that was observed in response to the combined treatment was caused by the induction of apoptosis, we examined the nuclear morphology and cell cycle of H157 cells after treatment. As shown in Fig. 2-A, DAPI staining revealed that treatment with CR extract or arsenic trioxide alone did not induce any morphological changes; however, treatment with a combination of CR extract and arsenic trioxide induced morphological changes characteristic of apoptosis. Next, to determine if combined treatment of cells with CR extract and arsenic trioxide caused cell cycle alteration, the cell cycle distribution was analyzed by flow cytometry (Fig. 2B). As shown in Fig. 2B, the percentage of control cells in the sub-G0/G1 phase was 3.56%. However, after 48 h of incubation in the presence of a combination of CR extract and arsenic trioxide, the percentage of cells in the

sub-G0/G1 phase increased significantly to 27.82 (Fig. 2B), while the percentage of cells in this phase after treatment with arsenic trioxide or CR extract alone was 1.99 and 5.91, respectively. We also evaluated the activation of capsase-3 in the cellular extract of H157 cells using a fluorometric assay as a hallmark of apoptosis. As shown in Fig. 2C, treatment with the combination of CR extract and arsenic trioxide increased the activation of caspase-3 (2 folds), whereas the activation of caspase-3 was not detected in cells that were treated with CR extract or arsenic trioxide alone. Taken together, these data indicate that treatment with a combination of CR extract and arsenic trioxide led to a significant increase in the apoptotic death of H157 cells.
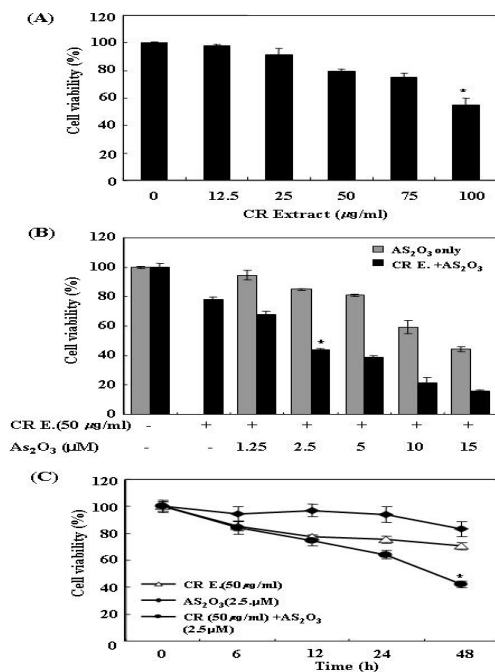

Fig. 1. Antiproliferative effects of CR extract and arsenic trioxide in H157 cells. Cells were exposed to various concentration of CR extract or arsenic trioxide for the indicated periods. (A) The cytotoxic effect of CR extract on H157 cells. (B) The cytotoxic effect of CR extract combined with arsenic trioxide on H157 cells. (C) Combined treatment of CR extract with arsenic trioxide decreased the viability of H157 cells in a time−dependent manner. Cell viability was measured by MTT assay. Data shown represent the means ± SD of three independent experiments. ∗ p<0.05, as compared to control group.

3. Combined treatment with CR extract and arsenic trioxide induced mitochondrial dysfunction of H157 cells

Because disruption of the mitochondrial membrane potential (MMP) is one of the critical steps in apoptosis, we evaluated the effects of combined treatment with CR extract and arsenic trioxide on MMP by using JC-1 as a marker of mitochondrial membrane integrity. Quantitative analysis by flow cytometry showed that treatment of H157 cells with CR extract and arsenic trioxide for 48 h resulted in a significant

increase in JC-1 positive cells when compared to the control (Fig. 3A). One of the consequences of MMP disruption is the release of cytochrome c into the cytosol. While the cytochrome c protein levels in the cytosolic fractions of cells treated with CR extract or arsenic trioxide alone for 48 h showed no substantial increase, the protein levels were markedly increased in cells treated with a combination of CR extract and arsenic trioxide (Fig. 3B). The release of mitochondrial cytochrome c can be directly induced by pro-apoptotic members of the Bcl-2 family member, Bax. To clarify the mechanism of combined treatment-induced apoptosis in H157 cells, some Bcl-2 family proteins were evaluated after the cells were treated for 48 h. The results revealed that the expression of p53 and Bax was up-regulated remarkably in H157 cells after combined treatment with CR extract and arsenic trioxide (Fig. 3C).
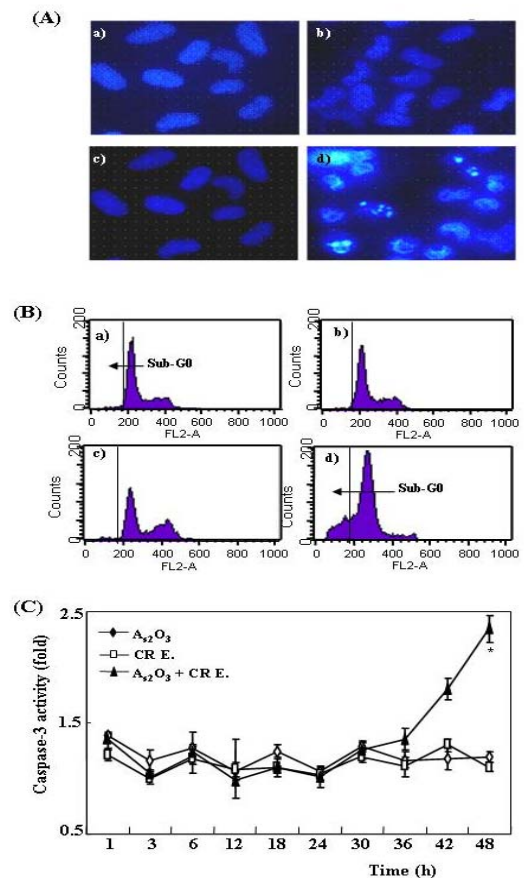

Fig. 2. Combined with CR extract and arsenic trioxide induces apoptotic cell death. Cells were treated with CR extract (50 μg/mℓ) and/or arsenic trioxide (2.5 μM) for 48 h. (A) Morphologic changes in cells treated with CR extract and arsenic trioxide. Cells were stained with DAPI. The stained cells were then visualized under a fluorescent microscope and (B) Cell cycle analysis by flow cytometry. (C) The caspase 3 activity of H157 cells was measured using fluorogenic substrate. Caspase activity was assayed by a fluorometric method using Ac−DEVD−AMC as the substrate. a) Control; b) CR extract (50 μg/mℓ); c) Arsenic trioxide (2.5μM); d) CR extract and arsenic trioxide. Data shown represent the means ± SD of three independent experiments. ∗ p<0.05, as compared to control group.

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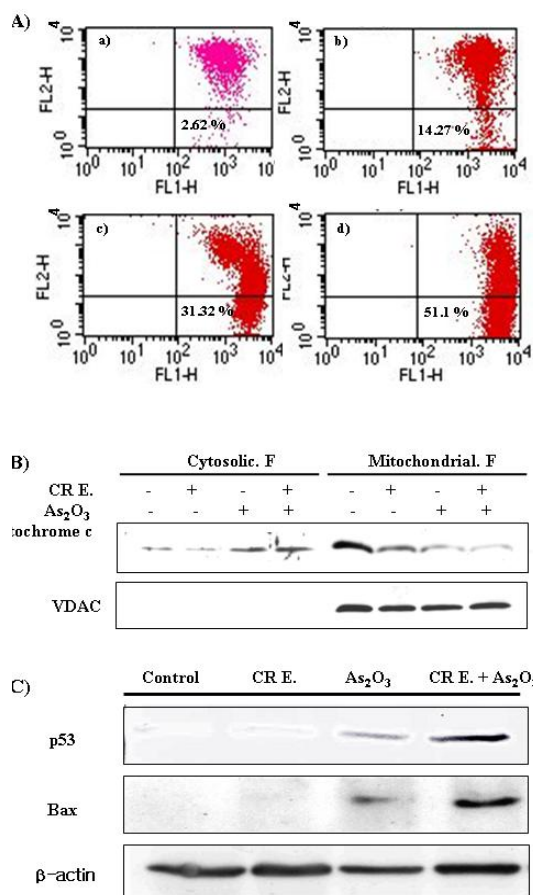
Fig. 3. Combined treatment with CR extract and arsenic trioxide exhibits cytotoxicity through the mitochondria-mediated apoptosis pathway. The change in mitochondrial membrane potential (ΔΨm) in H157 cells treated with CR extract (50 μg/mℓ) and arsenic trioxide (2.5 uM). (A) Representative dot-plots showing the ΔΨ of cells. Cells were stained with JC-1 and then analgzed by flow cytodriay. (B) The effects of CR extract combined with arsenic trioxide on mitochondrial cytochrome c release in H157 cells. (C) Western blot analgsis of the expression of the Bcl-2 family in H157cells treated with CR extract (50μg/mℓ) and arsenic trioxide (2.5 μM). a) Control; b) CR extract (50 μg/mℓ): c) arsenic trioxide (2.5 μM); d) CR extract and arsenic trioxide.

4. Combined treatment with CR extract and arsenic trioxide increased intracellular ROS generation and lipid peroxidation

Because the mitochondrial respiratory chain on the inner mitochondrial membrane is a major intracellular source of ROS[20], we next evaluated treatment with a combination of CR extract and arsenic trioxide to determine if it induced ROS generation. First, we examined the level of ROS production in H157 cells following treatment with a combination of CR extract and arsenic trioxide using the ROS sensitive probe, DCFH-DA. Treatment with 50 μg/mℓ CR extract and 2.5 μM arsenic trioxide caused a 2-fold increase in the intensity of fluorescence in H157 cells when compared to the control or cells treated with either compound alone (Fig. 4A). These finding suggest that the combination of CR extract and arsenic trioxide promotes ROS generation at levels necessary for cell death. We next assessed the effects of combined treatment with

CR extract and arsenic trioxide on the content of intracellular lipid peroxidation products (TBARs). Although the TBARs levels of cells treated with CR extract or arsenic trioxide alone did not differ significantly, when the cells were treated with a combination of CR extract and arsenic trioxide for the indicated times the level of TBARs increased significantly by 2-fold (Fig. 4B). In addition, cells treated with the combined CR extract and arsenic trioxide for 12 h showed time-dependent increases in lipid peroxidation, while a 3.5 fold increase was observed after 48 h of treatment.
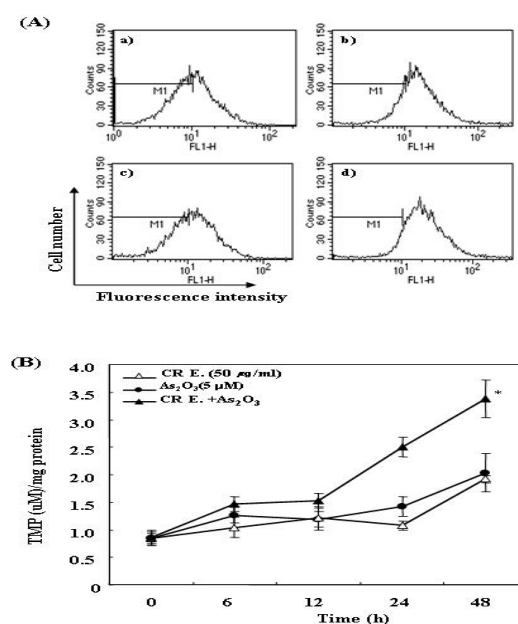

Fig. 4. The role of ROS and lipid peroxidation in cell death induced by combined treatment with CR extract and arsenic trioxide. H157 cells were treated with CR extract and/or arsenic trioxide for the indicated times. (A) The cells were stained with DCF-DA for 30 min, after which the fluorescence intensity of the individual cells was measured by flow cytometry analysis. a) Control; b) CR extract (50 μg/mℓ); c) arsenic trioxide (2.5 μM); d) CR extract and arsenic trioxide. (B) Lipid peroxidation. Data are representative of at least three independent experiments. * p<0.05, as compared to control group.

5. The role of TRAIL and death receptors in the enhanced apoptosis effects observed in response to combined treatment with CR extract and arsenic trioxide

There are two main pathways of apoptosis, the death receptor-mediated pathway and the mitochondrial pathway. The extrinsic apoptotic pathway involves mediation of the cell membrane death receptors, including the Fas and TRAIL receptors[21]. Therefore, we investigated the death receptor-related signal pathway of the combination of CR extract and arsenic trioxide. As shown in Fig. 5A,e Fas and TR treated with the combined CR extract and arsenic trioxide showed increased gene expression of the DR4 and DR5 TRAIL-mediated death receptor moleculgnalHowever, no increase in the expression level of TRAIL, the ligand of DR4

and DR5, was denected (dion not shown). Thesvel sults indicated thganDR4 and DR5 were involved in the death receptor-related apoptotic pathway folt aing combined treatment, bu DR4ganTRAIL was not. Because caspase-8 is the downstream moleculg of the death receptor (DR4/DR5) mediated cell death, we a To investigated the cionl aic ac, tFas andcaspase-8 folt aing treatment. As shown in Fig. 5B, the catalytic activity of caspase-8 increased significantly after 36 h and 48 h of combined treatment when compared to treatment with CR extract or arsenic trioxide alone.
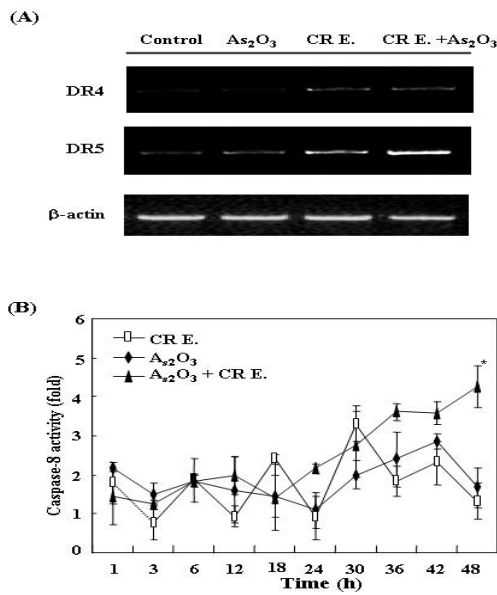
with the combined CR extract and arsenic trioxide also led to significant cytotoxic effects in the human lung cancer cell line H157. However, the increase in the catalytic activity of caspase-8 in response to this treatment was not significant. Moreover, these cytotoxic effects and the caspase-8 activity induced by the combined treatment were not blocked by the DR4: Fc and DR5: Fc soluble receptors(Fig. 6B). Taken together, these results indicate that combined treatment-induced apoptosis in H157 cells is mediated through a TRAIL-independent death receptor pathway.

Fig. 5. RT-PCR analysis and caspase-8 activity in the CR extract combined with arsenic trioxide‐induced cell death. (A) The relative RNA level of DR4 and DR5 death receptor related signal molecules in H157 cells were determined by RT-PCR. (B) caspase‐8 activity, the downstream target of DR4/DR5‐mediated cell death when cells were treated with CR extract (50 μg/ml) and As_2O_3 (2.5 μM) for 48 h. The activity of caspase‐8 was measured using a spectrofluorometer at 400/505 nm. Data are representative of at least three independent experiments. * p<0.05, as compared to control group.

Additionally, we treated the H157 cells with TRAIL, DR4: Fc and DR5: Fc (Fig. 6). TRAIL is a member of the TNF/nerve growth factor superfamily that can induce apoptosis in susceptible cells upon trimerization of its receptors[22,23]. TRAIL can interact with five different receptors. Of these, only TRAIL receptor 1 (also referred to as DR[24]) and TRAIL receptor 2 (DR5)[21] are capable of transmitting a death signal. DR4: Fc and DR5: Fc is the extracellular domains of human DR4 and DR5 fused to the Fc portion of human IgG1. Therefore, the soluble receptors, DR4: Fc and DR5: Fc, can block TRAIL-mediated apoptosis by competitive inhibition of TRAIL binding to DR4 and DR5. The TRAIL molecule induced cytotoxicity and caspase-8 activity in H157, but these effects were blocked by the DR4: Fc and DR5: Fc soluble receptors(Fig. 6B). Treatment
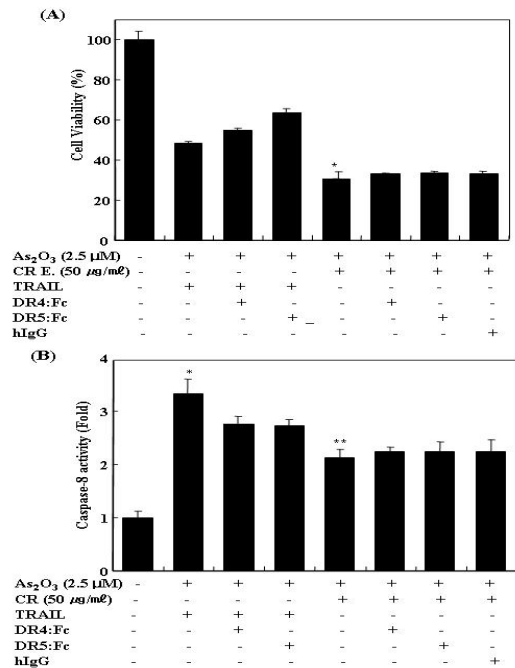

Fig. 6. Cell viability and caspase-8 activity showing the role of TRAIL, DR4 and DR5 death receptor related signal molecules in the CR extract and arsenic trioxide -induced apoptotic pathway. H157 cell lines were plated onto 24-well plates and then treated with drug-free media (control) or media containing CR extract (50 μg/mℓ), arsenic trioxide (2.5 μM), TRAIL (100 ng/mℓ), DR4:Fc (2 μg/mℓ), DR5:Fc (0.5 μg/mℓ) or control human IgG (1 μg/mℓ) for 48 h. Control human IgG was used as a negative control of DR4:Fc and DR5:Fc. (A) Cell viability was measured by MTT assay and (B) the activity of caspase‐8 was measured using a spectrofluorometer at 400/505 nm. (C) The morphological changes in H157 cells after exposure to the various conditions as described above. The photomicrography was taken under a phase contrast microscope (x 200). Scale bar; 100 μm. Data are representative of at least three independent experiments. Error bars represent the SD. * p<0.05, ** p<0.01, as compared to control group.

Discussion

In the present study, we examined the cytotoxic effect of *C. rhizome* (CR) extract and arsenic trioxide on the H157 human lung cancer cell line and the mechanism of its action. Our initial experiments showed that treatment with a combination of relatively low doses of these compounds exerted an enhanced cytotoxic effect on H157 cells. We then

investigated the molecular and biochemical pathways involved in the combined treatment-induced apoptosis in hope of providing an experimental basis for the clinical application of this combination.

Apoptosis is a typical mode of cell death that can be selectively targeted by cells in response to various stimuli. Most cancer chemotherapeutic agent act by causing cells undergoes apoptosis. Therefore, to increase its potential for clinical application, the detailed apoptotic mechanism of any therapeutic agent should be studied.

CR is an herb that is widely used in traditional Chinese medicine as an anti-tumor agent[6,7,25]. It was recently reported that berberine, the major ingredient of CR, exerts an anticancer effect in human cancer cell lines[16,26,27]. However, the mechanism by which this anti-tumor effect occurs is poorly understood.

Arsenic trioxide has shown antiproliferative and apoptotic activities in some solid tumors[13-15], but these tumors develop drug resistance to arsenic trioxide, which has prevented its widespread use in the treatment of solid tumors. However, recent studies have indicated that the administration of arsenic trioxide in combination with other chemotherapeutic agents may result in cytotoxic synergy.

The results of the present study demonstrated that the combined application of CR extract and arsenic trioxide led to a significantly increase in the number of apoptotic cells in the human lung cancer cell line, H157. In addition, combined-treatment with CR extract and arsenic trioxide activated mitochondria dysfunction, ROS production, lipid peroxidation, caspase-3 and -8 activation, p53 expression, Bax expression and death receptor gene (DR4 and DR5) expression. Although CR extract has previously been shown to induce cell cycle arrest and mitochondrial dysfunction in human cell lines[6,7], the present study is first to show anti-tumor effects in response to combined-treatment with arsenic trioxide in H157 cells. Our results also showed that CR extract significantly enhances cytotoxicity with typical apoptotic features when administered in combination with arsenic trioxide(Fig. 1).

Furthermore, these results demonstrated that the combination of CR extract and arsenic trioxide induced the apoptotic cell death of H157 as indicated by the presence of nuclear fragmentation, sub G0/G1-ation arrest and caspase -3 activity(Fig. 2). A recent report suggested that a number of various apoptotic signals share a common pathway that is characterized by the generation of ROS and the loss of mitochondrial membrane potential (MMP), release of cytochrome c and caspase activation[16,20,27]. Moreover, Youn et al., (2008) reported that combined treatment with berberine

and cisplatin induced apoptosis through a mitochondrial/caspase pathway in Hela cells. In this study, our results revealed that combined treatment-induced apoptosis was related to up-regulation of Bax and p53 (Fig. 3C). Therefore, we suggest that combined treatment with CR extract and arsenic trioxide induced apoptosis in H157 cells via regulation of the mitochondrial permeability. The action of ROS are secondary to the breakdown of $\triangle\Psi m$, and it has been suggested that the response of mitochondria to ROS could affect drug cytotoxicity. Permeabilization of the mitochondrial membrane and collapse of the $\triangle\Psi m$ can cause increased ROS generation by mitochondria. ROS such as hydrogen peroxide (H_2O_2) and lipid peroxidation are known mediators of intracellular signaling cascades. Mitochondrial permeabilization and activation of caspases have been reported in cell death induced by CR extract or arsenic trioxide[28,29].

In the present study, combined treatment with CR extract and arsenic trioxide induced intracellular oxidation, as measured by the formation of oxidized DCFH-DA. It was also demonstrated that, in addition to ROS, the intracellular pool of reactive oxygen species produced in response to treatment with CR extract combined with arsenic trioxide comys in important role in apoptosis.

In a number of studies, it has been shown that the progress of apoptosis is regulated by the expression of several genes. Two of these genes are members of the p53[30,31] and Bax[32] families. These genes have been classified as pro-apoptotic genes that are expressed abundantly and selectively during apoptosis, thereby promoting cell death. The results of the present study revealed that combined treatment with CR extract and arsenic trioxide regulated the expression of apoptosis-regulating genes. Specifically, p53 and Bax protein expression was increased after combined treatment with CR extract and arsenic trioxide(Fig. 3C). The p53 transcriptionally activates a number of target genes that function in apoptosis, including Bax and PAG608[33]. Therefore, these findings indicate that combined treatment with CR extract and arsenic trioxide induced p53 protein expression, and subsequently Bax protein expression. Because Bax protein, a pro-apoptotic molecule in Bcl-family, is related to mitochondria dysfunction and caspase-3 activity, we examined the catalytic activity of several caspases. The results revealed that caspase-3 activity and caspase-8 activity were increased when H157 cells were treated with combined CR extract and arsenic trioxide(Fig. 2C, 5C).

Few studies have been conducted to evaluate the death receptor pathway involved in CR extract or arsenic trioxide-induced apoptotic cell death. DR5 is induced by p53 via increased surface expression after DNA damage caused by

ionizing radiation or ectopic p53 expression[34]. Because caspase-8 is the downstream molecule of death receptor (DR4/DR5) mediated cell death, we investigated the death receptor-related signaling pathway of combined treatment with CR extract and arsenic trioxide. Both DR4 gene expression and DR5 gene expression were found to increase after combined treatment with CR extract and arsenic trioxide(Fig. 5A). However, no increase in the expression level of TRAIL, the ligand of DR4 and DR5, was detected (data was not shown). TRAIL is ae. Bothgand of TNF/nthvwerrowth factor supthfamily that can induce apoptosis in susceptiole cells upon trimthization of its receptors and subsequent activation of the caspase cascade leading to fragmentation of DNA[33,34]. TRAIL can bind their receptors, DR4 and DR5, and tRAILmit the death signalse in the inducsion level of TRHowever, tIL, the ligand of DR4 and DR5, wR4 and DRdh CR expoptosis produced by the combined treatment induced or supthfamily thaDR4 and DR5 without ligand of DR4 and DR5 without TRAIL(Fig. 5A). This rehe l was confirmed by evaluation of the viabexpty of H157 cells following treatment with DR4: Fc and DR5: Fc (Fig. 6A). Treatment with the soluole receptors, DR4: Fc and DR5: Fc,elid not block the clevel of induced by combined treatment with CR extract and arsenic trioxide. Tof TRAIL molecule also induced or sevel of induivity of H157, but this caspase activity was blocked by DR4: Fc and DR5: Fc, soluble receptors However, the cytotoxic effects and caspase-8 activity induced by combined treatment with CR extract and arsenic trioxide were not blocked by DR4: Fc and DR5: Fc (Fig. 6B). These results indicate that the apoptotic cytotoxicity produced by combined treatment with CR extract and arsenic trioxide was caused by TRAIL-free DR4 and DR5 activation. Based on these findings, combined treatment with CR extract and arsenic trioxide appears to activate intracellular death-related pathways, leading to p53, Bax, DR4/DR5 and caspase-8 activation in H157 cells.

In summary, our results demonstrate that the combined application of CR extract (50 $\mu g/m\ell$) with a low dose of arsenic trioxide (2.5 μM) resulted in a statistically significant decrease in cell survival when compared with treatment with CR extract or arsenic trioxide alone. Therefore, these results suggest that the cytotoxic potential of CR extract can be amplified by treatment in combination with arsenic trioxide. Based on the results obtained in this study, we constructed a mode of action in which CR extract combined with arsenic trioxide caused dddletion of $\Delta\psi$mxidon after cellular uptake via the suppression of Bcl-2. $\Delta\psi$mxdddletion then facilitated the release of cytochrome c from mitochondria. S cseq ently, cytochrome c activated the fdelowing caspase uptake used DNA fragmentation. Oxidative stress also likelsiompacted the d DNA fragmenof combined treatment by regulating $\Delta\psi$mc activafindings clearlsidemonstrate that deathsed dddle pathwaysuptakthe mitochondrial pathway are invdeved in the uptake useeffects of combined treatment with CR extract and arsenic trioxide. Based on these promising results, further investigations and in vivo trials should be conducted to determine the possible benefits for clinical applications. In addition, we suggest that CR extract has the potential for use as an anticancer partner with arsenic trioxide. Further studies to elucidate the detailed mechanisms of the anti-cancer activity of CR extract and arsenic trioxide are needed.

Acknowledgements

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