Methylene Chloride Fraction of *Spatholobi Caulis* Induces Apoptosis via Caspase Dependent Pathway in U937 Cells

Eun-Suk Ha, a Eun-Ok Lee, a Taek-Joon Yoon, a Jin-Hyung Kim, a Jong-Oh Park, b Nak-Cheol Lim, b Sung-Ki Jung, a Byung-Soo Yoon, a and Sung-Hoon Kim* a

a Department of Oncology, Graduate School of East-West Medical Science, Kyunghee University; Yongin 449–701, Korea; b Department of Internal Medicine, College of Oriental Medicine, Kyunghee University; Seoul 130–702, Korea; and Department of Biology, College of Natural Science and Engineering, Kyunggi University; Suwon 443–760, Korea. Received April 26, 2004; accepted July 2, 2004

*Spatholobi Caulis* has been used in Oriental medicine to treat cancer and blood stasis. In this study, the methylene chloride fraction of *Spatholobi Caulis* (MCSC) was examined to determine if it possesses anti-cancer activity via its apoptosis-inducing activity. MCSC exhibited a strong cytotoxic effect against human monocyte leukemia U937 cells (IC50=15.1 μg/ml). A TUNEL assay showed that the MCSC caused a characteristic ladder pattern of discontinuous DNA fragments and apoptotic bodies. Flow cytometric analysis confirmed that MCSC significantly increases the number of apoptotic cells stained by annexin V/PI cells. Western blotting revealed that MCSC activated caspase-3 expression and cleaved poly (ADP-ribose) polymerase (PARP) in a concentration-dependent manner. An enzyme-linked immunosorbent assay (ELISA) demonstrated that MCSC significantly activated the caspase-3 activity compared with the untreated control. Taken together, these results suggest that MCSC can induce apoptosis in U937 cells via the caspase dependent pathway.

Key words *Spatholobi Caulis*; U937; apoptosis; annexin V; caspase-3

Apoptosis is a physiological programmed cell death, which plays an important role in the development, maintenance of homeostasis and the elimination of unwanted or damaged cells from multicellular organisms.1) It can be triggered by numerous stimuli, including antigens, carcinogens, extracellular calcium, UV irradiation, growth factor depletion and chemotherapeutic agents.2,3) Apoptosis is characterized as a series of conserved steps. The early steps include chromatin condensation, nuclear membrane blebbing and cyttoplasmic shrinkage. The late steps include loss of adherence, DNA degradation, compacted organelles, condensed cytoplasm and nuclear material into membrane-bound apoptotic bodies and phagocytosis by the neighboring cells.3) Caspases, a family of cysteine proteases, are expressed in almost all cell types as inactive proenzymes.4) The activation of the caspases breaks down the cellular structure by the recognition and cleavage of specific proteins.5) Therefore, caspases have been identified as the major components of apoptosis.5) When cells receive apoptotic stimuli, cytochrome c release can be initiated from the mitochondrion, which then binds to the apoptotic protease activation factor (Apaf)-1. It activates caspase-3 via proteolytic processing5) and the cleavage of the DNA repair associated with nuclear enzyme poly (ADP-ribose) polymerase (PARP) to form a 85 kDa fragment, which has become a hallmark for apoptosis.4)

The induction of apoptosis in neoplastic cells is important in cancer treatment, because an impairment in apoptosis is related to cell immortality and carcinogenesis.7) There is evidence showing that many compounds isolated from crude drugs exert anti-tumoral activity by inducing apoptosis in cancer cells, e.g. ginseng saponin,7,8) curcumin from *Curcuma longa* L.,9) eupatilin from *Artemisia asiatica* Nakai (Asteraceae),10) costunolide from *Saussurea lappa* L. (Myrtaceae),10) the water-soluble ingredients of *Anemarrhena asphodeloides* Bunge,11) tritoli from *Tripterygium wilfordii*,12) and the 70% acetone extract from Eugenia jambos L. (Myrtaceae).13)

In Oriental medicine, the stem of *Spatholobi Caulis* has been used for the removal of blood stasis associated with menorrhagia, arthralgia and muscular pain.14) Blood stasis is associated with a cancer metastasis because a blood clot produced by platelets and tumor cells induces cancer metastasis.15) Previously, it was reported that the ethyl acetate fraction of *Spatholobi Caulis* blocked tumor cell-induced platelet aggregation and tumor cell invasion.16) However, it is possible that the methylene chloride fraction of *Spatholobi Caulis* may act differently from its ethyl acetate fraction. Therefore, this study investigated the cell death mechanism induced by the methylene chloride soluble fraction of *Spatholobi Caulis* (MCSC) against human monocyte leukemia U937 cells.

MATERIALS AND METHODS

**Plant Materials** *Spatholobi Caulis* is a dried stem of *Spatholobus suberecetus Dunn* which was purchased from Daehyo Pharmaceutical Co. (Seoul, Korea) and kindly authenticated by Dr. Deok-Kyun Ahn (Kyunghee univ.). The botanical origin, voucher number, harvesting season and harvesting place of *Spatholobi Caulis* are *Spatholobus suberecetus Dunn*, GSM0005, autumn 2000 and South Korea, respectively. The methylene chloride (MC) fraction was prepared according to following procedures. The dried stem of *Spatholobi Caulis* (600 g) was cut into pieces and extracted with methanol (MeOH, 2 l) for 1 week at room temperature. After filtration, the MeOH extract was concentrated using a rotary vacuum evaporator (EYELA N-1N, Tokyo Rikakikai Co., Ltd., Japan) and lyophilized (Freeze dryer Lioalfa-6, Telstar, Spain) to produce 43 g of the MeOH extract of *Spatholobi Caulis*. The MeOH extract was dissolved in distilled water and fractionated with n-hexane (Hx), MC, ethyl acetate (EA) and butanol (BuOH), successively, to give the
soluble fractions and finally a water-soluble fraction for biological test. The selected MC fraction of Spatholobi caulis (MCSC) was concentrated and lyophilized. The yield of the MC fraction was 3.56 g (0.6% of the starting material) in terms of the dried medicinal herb (Fig. 1).

**Cell Culture** The human monocyte leukemia U937 cell line (ATCC CRL-1593.2) was obtained from American Type Culture Collection (ATCC, VA, U.S.A.). They were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere of 5% CO₂/95% O₂ air.

**Cytotoxicity Assay** The cytotoxicity of the MCSC against U937 cells was determined by XTT assay. Briefly, U937 cells were seeded on 96-well culture plates at a density of 2×10^4 cells per well in 100 µl of the culture medium containing with or without various concentrations of MCSC. After 6, 12 or 24 h incubation, respectively, 50 µl of XTT solution was added to each well, and the cultures were incubated for a further 4 h. The absorbance of the plates was measured at 450 nm using microplate reader (Molecular Devices Corp., CA, U.S.A.).

**DNA Gel Electrophoresis** Untreated and MCSC-treated U937 cells (2×10^6 cells) were harvested and washed with PBS. The cells were resuspended in 500 µl of lysis buffer [10 mM Tris, pH 7.8, 2 mM EDTA, 0.5% SDS] and incubated for 10 min at room temperature. The supernatant was collected and incubated with 2 µl of 20 mg/ml RNase A for 1 h at 37 °C. After treatment with 2 µl of proteinase K (20 mg/ml) for 3 h at 56 °C, genomic DNA was extracted with 1 volume of phenol/chloroform/isoamylalcohol (25 : 24 : 1, v/v, Sigma, MO, U.S.A.) solution, and DNA was precipitated by incubating with 0.1 volume of 3x NaOAc and 2.5 volume of cold 100% alcohol. DNA pellet was washed with 70% ethanol, dried under vacuum and subsequently dissolved in 25 µl of TE buffer [1x Tris-Cl, 100 mM EDTA, pH 7.5]. DNA samples were subjected to 1.5% agarose gel and the bands were visualized under ultraviolet illumination.

**Measurement of Apoptotic Cells by Flow Cytometry** For flow cytometric analysis of sub G1 cell with fragmented DNA, 5×10^5 cells/well onto 6-well plates were collected at different concentrations of MCSC. The cells were harvested and incubated with 1 ml of 75% cold ethanol for 2 h at −20 °C and then washed with PBS. Cell pellets were incubated with 10 µg/ml RNase before adding 50 µg/ml propidium iodide. Samples were analyzed by FACSort flow cytometer using the Cell quest analysis program (Becton Dickinson, San Jose, CA, U.S.A.), which is also used to determine the percentage of apoptotic cells. PI was excited at 488 nm, and fluorescence was analyzed at 620 nm.

**TdT-Mediated dUTP Nick-End Labeling (TUNEL) Assay** Terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL)-positive cells were detected using DeadEnd™ Fluorometric TUNEL SYSTEM (Promega, WI, U.S.A.) according to the manufacturer’s instructions. Briefly, U937 cells were treated with MCSC or camptothecin (CPT) and the cells were washed and harvested after 12 h. Cells were fixed with 4% methanol-free formaldehyde solution for 25 min at 4 °C and added with 0.2% Triton X-100 solution for 5 min at 4 °C. Intracellular DNA fragments were then labeled by exposing cells to fluorescein-12-dUTP and terminal deoxynucleotidyltransferase for 1 h at 37 °C, in a humidified atmosphere and protected from light. The reaction was stopped by adding SSC solution, followed by washing the cells three times and stained with 1 µg/ml PI solution in PBS containing 250 µg/ml DNase-free RNase for 15 min at room temperature in the dark. After washing with PBS, slides were mounted in 30% glycerol in PBS and analyzed under a Axiovert S 100 fluorescence microscope (Carl Zeiss, Inc., U.S.A.).

**Western Blotting Detection Reagents (Amercham Biosciences, NJ, U.S.A.). The membranes were blocked with blocking solution containing 5% skim milk in PBS for 2 h at room temperature. The membranes were then probed with anti-caspase-3 (Santa Cruz Biotechnology Inc., CA, U.S.A.) and anti-PARP (Pharmingen, MA, U.S.A.) antibodies. The primary antibodies were detected using horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Pharmingen, MA, U.S.A.), and visualized by enhanced chemiluminescence (ECL) Western Blotting Detection Reagents (Amercham Biosciences, NJ, U.S.A.).**

**Caspase-3 Colorimetric Assay** For detection of caspase-3 activity, U937 cells were treated with MCSC or CPT for 12 h. The cells were collected and cells were induced by addition of 50 µl of lysis buffer following incubation on ice for 10 min. After collection of protein, the quantity of protein was measured by DC protein assay kit (Bio-Rad Laboratories, CA, U.S.A.) with bovine serum albumin (BSA) as the standard. The samples (20 µg of total protein) were mixed with 5X SDS sample buffer [0.3 M Tris–HCl (pH 6.8), 25% 2-mercaptoethanol, 12% SDS, 25 mM EDTA, 20% glycerol, and 0.1% bromophenol blue] and the mixtures were boiled at 95 °C for 5 min. They were subjected to SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Amersham Biosciences, NJ, U.S.A.). The membranes were blocked with blocking solution containing 5% skim milk in PBS for 2 h at room temperature. The membranes were then probed with anti-caspase-3 (Santa Cruz Biotechnology Inc., CA, U.S.A.) and anti-PARP (Pharmingen, MA, U.S.A.) antibodies. The primary antibodies were detected using horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Pharmingen, MA, U.S.A.), and visualized by enhanced chemiluminescence (ECL) Western Blotting Detection Reagents (Amercham Biosciences, NJ, U.S.A.).

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mean values from three consecutive experiments and expressed as percentages according to the following equation:
The activity (%) = (mean absorbance of MCSC or CPT exposed wells – mean absorbance of blank wells)/(mean absorbance of control wells – mean absorbance of blank wells)×100.

High Performance Liquid Chromatograph (HPLC) Analysis The chromatographic system consisted of a pump (JAI Co. LTD, L-6000 pump), a UV detector (JAI Co. LTD, VU-310AB turnable absorbance detector) and a data modular (Yullin Technology, Korea; Networked Data Acquisition NDA-401). A phenomenex C18 column (4.6×250 mm; U.S.A.) was used. Acetonitrile-HPLC grade was purchased from Merck (Germany). Methanol- and Water-HPLC grade were purchased from Fisher Scientific Korea. Detection of the peaks of MCSC was at 225 nm. The injection volume was 10 µl and flow rate was 1.0 ml/min. Standard solution was filtered through 0.45 µm membrane filter and applied to HPLC.

Statistical Analysis All values were expressed as means±S.D. Statistical significance was compared between each treated group and control by the Student’s t-test. Data with p<0.05 were considered significantly different from untreated control.

RESULTS

Effect of MCSC on the Cytotoxicity on U937 Cells Cytotoxicity effect of MCSC was studied on U937 cells at concentrations ranging from 2.5 to 160 µg/ml for 6, 12, and 24 h. As shown in Fig. 2, the growth of the cells was significantly inhibited in a concentration-dependent manner. The values of IC50 of MCSC against U937 cells were 134.5 µg/ml at 6 h, 77.8 µg/ml at 12 h, and 15.1 µg/ml at 24 h, respectively.

Effect of MCSC on the Morphological Changes of U937 Cells To determine the apoptotic mode of MCSC in U937 cells, we examined whether MCSC could induce inter-nucleosomal degradation of DNA, a characteristic hallmark of apoptosis. U937 cells were treated with 10, 20, 40 and 80 µg/ml of MCSC for 12 h and the typical characteristics of cell death were verified by DNA fragmentation assay. As shown in Fig. 3A, a ladder pattern of discontinuous DNA fragments was displayed from 20 µg/ml of MCSC and 0.5 µM of CPT was used for positive control. Likewise, when U937 cells were treated with MCSC, we also observed many TUNEL-positive cells indicating small, dense and fragmented morphology with green fluorescence (arrows), while PI stained nucleus in control U937 cells showed round morphology with red fluorescence (Fig. 3B).

Effect of MCSC on the Annexin V Positive Cells in U937 Cells Annexin V binding analysis was performed to identify apoptosis. U937 cells were treated with various concentrations of MCSC for 12 h and then stained with FITC-conjugated annexin V. Propidium iodide (PI) staining was performed for the purpose of excluding necrotic cells. As shown in Fig. 4, MCSC increased the number of annexin V+/PI− cells in a concentration-dependent manner.

Effect of MCSC on Caspase-3 and PARP in U937 Cells Caspases are believed to play a central role in the apoptotic signalling pathway and contribute to the overall apoptotic morphology by cleavage of various cellular substrates. Therefore, we examined the activation of caspase-3 and proteolytic cleavage of PARP by treatment of MCSC for 12 h in U937 cells. MCSC significantly increased caspase-3 activity by ELISA (Fig. 5B) and down-regulated the expression of procaspase 3 by western blotting in a concentration-dependent manner. MCSC also caused the proteolytic cleavage of PARP in a concentration-dependent manner by western blot analysis (Fig. 5A).
The MCSC was analyzed by using HPLC. Chromatogram of MCSC was shown in Fig. 6.

**DISCUSSION**

One of the attractive strategies considered in current cancer chemotherapy is dietary or pharmaceutical manipulation aiming inducing the death of malignant cells via apoptosis.\(^7\)–\(^9\) Many reports have shown that herbal medicines as well as compounds isolated from natural products have potential anti-tumor activity by inducing apoptosis.\(^7\)–\(^9\),\(^14\) There is also evidence showing that naturally occurring compounds and many chemotherapeutic agents with anti-tumor effects can trigger the apoptosis of cancer cells.\(^20\),\(^21\) The stem of *Spatholobi Caulis* has been used in Oriental medicine to treat blood stasis and cancer.\(^15\) It was previously reported that the EA soluble fraction of *Spatholobi Caulis* may have anti-metastatic activity by inhibiting tumor cell-induced platelet aggregation and migration.\(^15\) In contrast, a different fraction from the same plant, the methylene chloride fraction of *Spatholobi Caulis* have a different TLC pattern (data not shown) from the EA fraction suggesting that MCSC may act differently from the EA fraction of *Spatholobi Caulis*. The HPLC profile of MCSC indicates that there are various components in MCSC. MCSC exhibited the strongest cytotoxicity against various cancer cell lines among the various solvent fractions such as methanol, hexane, methylene chloride, ethylacetate...
and butanol fractions (data not shown). Therefore, the effect of MCSC on apoptosis-inducing activity was examined. MCSC caused the characteristic morphological changes of apoptosis such as nuclear chromatin condensation, the loss of plasma membrane asymmetry, the activation of proteases and endonucleases, DNA degradation and the segmentation of U937 cells. This demonstrates that MCSC has apoptosis inducing activity. MCSC had strong cytotoxicity against U937 cells in a concentration-dependent manner. A DNA fragmentation assay revealed that MCSC could degrade the chromosomal DNA into small nucleosomal fragments. Unlike detection by the above-mentioned DNA ladder on agarose gels, TUNEL assay can detect apoptosis at the single-cell level thereby permitting a better evaluation of the apoptotic cells. PI cannot enter cells with intact membranes and is used to differentiate between the early apoptotic (Annexin V positive, PI negative) and late apoptotic or necrotic cells (Annexin V–PI double positive). Exposing the U937 cells to MCSC resulted in an increase in the early apoptotic cell population in a concentration-dependent manner. These results demonstrated that MCSC can induce apoptotic cell death in U937 cells.

Caspase-3, a cystein protease, is widely distributed in cells in the inactive form. Its activation requires the cleavage of the 32 kDa protein into its active subunits. In order to determine if caspase-3 is involved in the apoptosis induced by MCSC, cleavage of procaspase-3 was examined by western blot analysis. MCSC activated the cleavage of procaspase-3 in a concentration-dependent manner, and ELISA showed that the caspase 3 activity was significantly increased by MCSC, indicating that caspase 3 is involved in MCSC induced apoptosis. Another feature of apoptotic cell death is the cleavage of the 85 kDa PARP producing fragments in response to caspase-3 activation. MCSC effectively cleaved PARP suggesting that MCSC induces apoptosis in the U937 cells via a caspase-dependent pathway.

In summary, MCSC exhibited strong cytotoxicity against U937 cells. It significantly increased the subG1 cell population and showed apoptotic bodies by TUNEL assay and DNA fragmentation assay. MCSC activated caspase 3 and cleaved PARP. Overall, these results suggest that MCSC can induce apoptosis via a caspase dependent pathway. However, in order to further examine the apoptotic mechanism it will be necessary to isolate effective single compound from the methylene chloride fraction of Spatholobi Caulis.

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REFERENCES