Asiatic Acid Induces Colon Cancer Cell Growth Inhibition and Apoptosis through Mitochondrial Death Cascade

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Abstract

Cancer is one of the leading causes of death in the world. The triterpenoid compound asiatic acid derived from the tropical medicinal plant Centella asiatica displays cytotoxic activity on fibroblast cells and several other kinds of cells. The present work studies asiatic acid-mediated growth inhibition of cancer cells and the underlying mechanism. Asiatic acid markedly inhibited cancer cell proliferation. Apoptosis of SW480 human colon cancer cells was induced by asiatic acid as shown by flow cytometry, DNA fragmentation and nuclear chromatin condensation experiments. Through increasing mitochondrial membrane permeability and cytochrome c release from mitochondria into cytosol, asiatic acid induced caspase-9 activity, which further activated caspase-3 and poly(ADP-ribose) polymerase cleavage resulting in irreversible apoptotic death in the tumor cells. Taken together, these results suggest that mitochondria death apoptosis cascade plays very important roles in asiatic acid-induced cancer apoptosis.

Key words asiatic acid; colon cancer cell; apoptosis; mitochondrial death cascade

Cancer, especially colorectal cancer, is one of the leading causes of death in the world. In the United States, colorectal cancer has become the second most common cause of cancer-related deaths.1 In 2008, an estimated 148,810 cases of colorectal cancer will be diagnosed and 49,960 people will die from this disease.2 Although there have been advances in knowledge regarding the biology of the disease, the treatment options for advanced colon cancer are limited and chemotherapy is still one of the most important strategies to prevent its development.

Triterpenes are bioactive tetracyclic or pentacyclic compounds that are present in many medicinal herbs. Their biological activities have attracted much attention.3 Several triterpenes have been reported to be cytotoxic to various tumor cell lines including human leukemia, neuroblastoma, and melanoma cells.4–6 Asiatic acid is a pentacyclic triterpenoid derived from the tropical medicinal plant Centella asiatica (C. asiatica), which displays cytotoxic activity on fibroblast cells.7 Asiatic acid induces apoptosis in human hepatoma cells due to Ca2+ release and p53 up-regulation.8 C. asiatica extract can inhibit the formation of azoxymethane-induced aberrant crypt foci and tumorigenesis in the rat colon9 and asiatic acid from C. asiatica can inhibit growth of HT-29 colon cancer cells by affecting Bcl-2 and Bcl-xL correlation.10 Asiatic acid induction of apoptosis in SK-MEL-2 human melanoma cells can occur upon increasing intracellular reactive oxygen species (ROS) level, which enhances the expression of Bax, but not Bcl-2 protein, in the melanoma cells.11

In present study, we evaluated the effects of asiatic acid on cancer cell lines and clarified the mechanism of the effects. Our results show that asiatic acid is able to inhibit proliferation and cause cell death that results from asiatic acid-mediated induction of apoptosis in SW480 cells. As well, we confirmed that asiatic acid can induce apoptosis mainly through interference with mitochondrial membrane potential, which stimulates the release of cytochrome c and triggering of caspase signaling pathways and poly(ADP-ribose) polymerase (PARP) activation, which in turn leads to apoptotic cell death. Our results illuminate the anti-cancer mechanism of asiatic acid, which may shed further light on its potential chemopreventive application against cancers.

MATERIALS AND METHODS

Compounds Asiatic acid (Fig. 1A) previously isolated2 from the urban of Centella asiatica (Umbelliferae) was used. The compound used for this study was checked by high-performance liquid chromatography and was shown to be >97% pure. In the present study, asiatic acid was dissolved in dimethylsulfoxide. The final concentration of solvent was always less than 0.1%.

Cell Culture and Reagents SW480 human colon cancer cells, SNU668 human stomach cancer cells and CT26 murine colorectal adenocarcinoma cell line derived from BALB/c mice were obtained from the Korean Cell Line Bank. They were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml). Cells were incubated at 37 °C in a humidified atmosphere with 5% CO2 and subcultured after trypsinization (Gibco, Franklin Lakes, NJ, U.S.A.).

Cell Proliferation Assay Cell proliferation assay was carried out using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega Corp., U.S.A.) as instructed by the manufacturer. Briefly, after 24 h seeding, cancer cells were replaced with fresh medium containing various concentrations of asiatic acid (0—50 μg/ml) for 24 h. Twenty microliters of methanethiosulfonate/phenazine methosulfate solution (MTS) was added to each well and incubated for 1—1.5 h at 37 °C. The absorbance was read at an optical den-
sity (OD) 490 nm using a Tecan Infinite F200 microplate reader (Tecan, Männedorf, Switzerland). SW480 cells were additionally treated with 20, 30, and 40 μg/ml asiatic acid for 3 h, 6 h, 9 h, 12 h, and 24 h. The inhibitory rates were calculated as \( \left[ 1 - \frac{OD_{\text{treated}}}{OD_{\text{control}}} \right] \times 100\% \), and the proliferation rates were calculated as \( \left[ \frac{OD_{\text{treated}}}{OD_{\text{control}}} \right] \times 100\% \).

Cell Cycle Analysis Propidium iodide staining was used to analyze DNA content and cell cycle distribution. After cells were exposed to different concentrations of asiatic acid for 24 h or a defined concentration of asiatic acid (30 μg/ml) for various times, SW480 cell morphology changes were assessed using a CKX41 phase-contrast microscope (Olympus, Tokyo, Japan). Both the adherent and floating cells were harvested, fixed with 70% ethanol, and incubated with 50 μg/ml propidium iodide (Sigma-Aldrich, St. Louis, MO, U.S.A.) and 1 μg/ml RNase A (Sigma-Aldrich) at 4°C in the dark for 30 min. The cells were then analyzed by flow cytometry using CellQuest software (Becton Dickinson, Franklin Lakes, NJ, U.S.A.). Apoptotic cells were considered to constitute the sub-G1 population and the percentage of nonapoptotic cells in each phase of the cell cycle was determined and analyzed using ModFit LT software (Verity Software, Topsham, ME, U.S.A.). All experiments were repeated and yielded similar results.

Fluorescence Microscopy Assay Nuclear DNA changes were observed by fluorescence microscopy stained with 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, U.S.A.). After treatment with different concentrations of asiatic acid for 24 h or 30 μg/ml asiatic acid for various times, SW480 cells were collected, fixed with 4% paraformaldehyde and stained with DAPI. Stained nuclei were visualized using an IX71 fluorescent microscope (Olympus). Blebbing of the membrane, chromatin aggregation and nuclear condensation were used as criteria to identify cells undergoing apoptosis.

DNA Laddering Assay DNA fragments were extracted as previously described. Briefly, after treatment with asiatic acid, SW480 cells were lysed and suspended in Tris–ethylenediaminetetraacetic acid (EDTA) buffer (pH 7.5). Precipitation solution was added, mixed and each sample was placed on ice for 15 min. Thereafter, the mixture was spun for 15 min at 14000×g and DNA in the supernatant was collected using a QIA-prep miniprep spin column (Qiagen, Valencia, CA, U.S.A.) and eluted with Tris–EDTA buffer (pH 8.0). The DNA samples were separated by electrophoresis on 1.5% agarose gels containing 1 μg/ml ethidium bromide (EB). Images were visualized under ultraviolet light using a SL-20 Image Visualizer EL Logic 100 gel documentation system (Seoulin Scientific, Seoul, Korea) and captured using Kodak ML software.

Mitochondrial Membrane Potential Assay The mitochondrial probe rhodamine 123 (Sigma-Aldrich) was used to detect the mitochondrial membrane potential changes. Briefly, after treatment with asiatic acid, SW480 cells were harvested, washed in phosphate buffered saline (PBS) and incubated with rhodamine 123 dye at 37°C in a 5% CO2 incubator for 30 min. The cells were then washed, resuspended in PBS and analyzed by flow cytometry. The percentage of cells that had lost mitochondrial membrane potential was calculated using CellQuest software (BD, Franklin Lakes, NJ, U.S.A.).

Determination of Cytochrome c Release The method for preparation of cytosolic fractions from cells was modified from previously described methods. Briefly, after treatment with asiatic acid, SW480 cells were collected and washed with PBS. Cell pellets were resuspended in ice-cold buffer A (20 mmol/l N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid (HEPES) pH 7.5, 10 mmol/l KCl, 15 mmol/l MgCl2, 1 mmol/l EDTA, 1 mmol/l ethylene glycol bis(2-aminoethyl ether)-N,N,N',N''-tetraacetic acid (EGTA), 1 mmol/l dithiothreitol, 5 μg/ml pepstatin A, 10 μg/ml leupeptin, 2 μg/ml aprotinin, and 0.1 mmol/l phenylmethylsulfonyl fluoride), allowed to swell on ice for 30 min, and centrifuged at 20000×g at 4°C for 20 min. The resulting supernatant fraction served as the soluble cytosolic fraction and was detected by Western blot.

Protein Extraction and Western Blot After treatment with asiatic acid, SW480 cell proteins were obtained by cell lysis in ice-cold RIP A buffer (50 mm Tris–HCl pH 7.4, 150 mm NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate). Total cell proteins and the cytosolic fraction obtained in the previous section were subjected to Western blot analysis.
electrophoresis on 8—12% polyacrylamide gels, transferred to nitrocellulose membranes and probed with the following antibodies: caspase-9, caspase-3, poly(ADP-ribose) polymerase (PARP) (Cell Signaling Technology, Beverly, MA, U.S.A.), and anti-cytochrome c antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). The immunoblots were developed and visualized by an enhanced chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ, U.S.A.). β-Actin was used as internal control.

**Statistical Analysis** Data presented represents the mean±S.D. of three independent determinations. All experiments were done at least three times, with three or more independent observations each time. Statistical analysis was performed using SAS 8.0 software. Significant differences were established at p<0.05.

RESULTS

**Asiatic Acid Inhibits Growth of Cancer Cells** To study the effect on cancer cells, cells were treated with various concentrations of asiatic acid (0—50 μg/ml). After treatment with asiatic acid for 24 h, growth of SW480, SNU668 and CT26 cells was all markedly inhibited (Fig. 1B). For SW480, asiatic acid showed the concentration- and time-dependent manner. When treated by 20, 30, 40 μg/ml asiatic acid, SW480 cell proliferation was inhibited beginning at 6 h and the effects were more significant until 12 h and 24 h (Fig. 1C).

**Asiatic Acid Induces Apoptosis of SW480 Cells** The morphologies of SW480 cells treated by asiatic acid were observed by phase-contrast microscopy. Compared with the adherent and spindle-shaped control cells, the morphology changes of drug-treated cells were notable: cells lost their adherent phenotype and assumed a circular morphology (Figs. 2A, C). To ascertain the mechanism of asiatic acid inhibition effects on colorectal carcinoma cells, SW480 cells were analyzed by flow cytometry. The cycle distributions of SW480 displayed unremarkable changes except the sub-G1 peaks. Their percentages changed from 1.4% in control cells to 3.4—43% in asiatic acid-treated cells at 24 h (Fig. 2B). When treated with 30 μg/ml asiatic acid, the percentages of sub-G1 peaks increased with time, reaching 25% by 24 h (Fig. 2D). One of the apoptosis events is the condensation of nuclear chromatin. Therefore, the morphology of asiatic acid-treated SW480 cells was investigated using DAPI staining. Cells treated with asiatic acid displayed the typical apoptotic nuclear morphology (nuclear shrinkage, DNA condensation and fragmentation) in a concentration-dependent manner, whereas the nuclear morphology was intact and normal.

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Fig. 2. Effects of Asiatic Acid on SW480 Cell Morphology and Cells Cycle
After treated by asiatic acid, cell morphologic changes were assessed using a phase-contrast microscope and the cell cycle changes were analyzed by flow cytometry. The morphologies (400×) (A), DNA histograms and cell cycle distribution (B) of SW480 cells were assessed after exposure to various concentrations of asiatic acid for 24 h. The morphologies (400×) (C), DNA histograms and cell cycle distribution (D) of SW480 cells were assessed after exposure to 30 μg/ml asiatic acid for various times. The percentages of cell cycle distribution correspond to their respective DNA histograms. Results are expressed as the representation of at least three independent experiments.
in the non-treated controls (Fig. 3A). As shown in Fig. 3B, nuclear shrinkage was evident at 3 h, and increased with time. Moreover, the apoptotic pattern was further revealed by DNA gel electrophoresis. As shown in Figs. 3C and D, the classic laddering pattern of inter-nucleosomal DNA fragmentation was observed in asiatic acid-treated cells, indicating that an irreversible apoptotic death had been induced.

**Asiatic Acid Strongly Induces Loss of Mitochondrial Membrane Potential and Release of Cytochrome c** The intrinsic apoptotic pathway involves mitochondria dysfunction caused by mitochondrial membrane permeabilization due to a loss of membrane potential. Rhodamine 123 is cell membrane permeable and accumulates in mitochondria with active membrane potential and fluorescent emission increases due to dye stacking. The stain intensity decreases when agents disrupt mitochondrial membrane potential. Asiatic acid induced loss of mitochondrial membrane potential in concentration- and time-dependent manners (Figs. 4A, B). The percentage of cells with loss of mitochondrial membrane potential increased from 2.7% in control cells to 5.7—42% after treatment with various concentrations of asiatic acid for 24 h (Fig. 4A). After treatment with 30 µg/ml asiatic acid for 3 h, the percentage of cells with loss of mitochondrial membrane potential had increased to 10% and increased continu-
ally to reach 27% by 24 h (Fig. 4B). Cytochrome c plays an important role in apoptosis. An apoptotic stimulus triggers the release of cytochrome c from the mitochondria into the cytosol. To further examine the role of mitochondria in induction of apoptosis by asiatic acid, we then measured the extent of mitochondrial release of cytochrome c using a subcellular fractionation method. As shown in Fig. 4C, 24 h treatment of SW480 cells with asiatic acid induced release of cytochrome c from mitochondria to the cytosol in a concentration-dependent manner. After treatment with 30 μg/ml asiatic acid for various times, the induced release of cytochrome c was evident at 3 h, and to a greater extent at 9—24 h (Fig. 4D). Taken together with the data on loss of mitochondrial membrane potential, these results suggest that the mitochondrial pathway plays an important role in the apoptosis induced by asiatic acid.

**Activation of Caspase Activity and PARP Cleavage by Asiatic Acid** Caspases are central components in the induction of apoptosis by various agents. They are activated from proforms to functional forms by partial cleavage events. When cytochrome c is released, it can bind to Apaf-1 and further activate caspase-9, which in turn activates caspase-3. Caspase-3 is a prevalent caspase that is ultimately responsible for the majority of apoptotic processes. It causes the cleavage or degradation of several important substrates including PARP. Therefore, we examined the effects of asiatic acid on the activation of caspase-9 and caspase-3 activation, and PARP cleavage by Western blot using specific antibodies that recognize the particularly cleaved and activated forms. Treatment of SW480 cells with asiatic acid for 24 h induced the activation of the caspases, and the cleavage of PARP in a concentration-dependent manner (Fig. 5A). Activation of caspase-9 and caspase-3 as well as its endogenous substrate PARP increased by 3 h and even more significantly by 24 h (Fig. 5B). These results suggest that caspase-9, caspase-3 and PARP play the main roles in asiatic acid-mediated induc-
tion of apoptosis.

DISCUSSION

Despite recent advancements in understanding the carcinogenic processes of colon cancer, the increasing incidence and relatively low remission rate of chemotherapy have spurred the scientific community to establish more effective treatment regimens by adopting novel and innovative approaches. The discovery and use of active medicinal compounds from herbal/natural sources have provided alternative treatment choices for patients. In the present study, we have shown that asiatic acid, one of the triterpenoids derived from the tropical medicinal plant Centella asiatica, has inhibitory effects on the growth of SW480 human colon cancer cells in a concentration- and time-dependent manner.

Apoptosis is a cell suicide program that is essential for the development and maintenance of tissue homeostasis and the elimination of unwanted or damaged cells from multi-cellular organisms. Thus, induction of apoptosis in tumor cells has been considered as a protective mechanism against development and progression of cancer. During apoptosis, cells lose their adherent and spindle-shaped phenotype, and assume a circular morphology; this was confirmed in the present study. To examine the mechanism that might account for the effects of asiatic acid in colon cancer cells, we first investigated its effects on cell cycle distribution. In flow cytometric histograms, apoptotic cells will show DNA fluorescence in sub-diploid regions with DNA content less than those in the G1 phase, and the percentage of the sub-G1 peak can subsequently be calculated. As shown in the results, the sub-G1 population in drug-treated groups increased with increasing concentration of asiatic acid or increasing time of use of asiatic acid. Apoptosis is characterized by a series of morphological changes involving cell shrinkage, chromatin condensation and the formation of apoptotic bodies. The morphological changes could also be observed in asiatic acid-treated SW480 cells. Moreover, the apoptotic pattern was further revealed by DNA gel electrophoresis. Therefore, from all of the aforementioned apoptosis-related experiments, we have confirmed that asiatic acid inhibits colon cancer cell proliferation mainly by inducing characteristic apoptosis. Our results are consistent with other reports of asiatic acid-mediated cytotoxic activity via the induction of apoptosis.

The mechanisms of apoptosis mainly involve two signaling pathways: the mitochondrial pathway and the cell death receptor pathway. The key element in the mitochondrial pathway is the efflux of cytochrome c from the mitochondria to the cytosol. Once cytochrome c is released into the cytosol, cytochrome c together with Apaf-1 activates caspase-9, and the latter then activates caspase-3. In the present study, the rapid loss of mitochondrial membrane potential and release of cytochrome c were observed in asiatic acid-treated colon cancer cells. Furthermore, we found that asiatic acid induced apoptosis by increasing the activation of caspase-9 and -3 activities. Moreover, caspase-3, a prevalent caspase ultimately responsible for the majority of apoptotic processes, causes the cleavage or degradation of several important substrates, including PARP. Intact PARP can help cells to maintain their viability, but cleavage of PARP facilitates cellular disassembly and serves as a marker of cells undergoing apoptosis. Increased cleavage of PARP was also presently observed after treatment by asiatic acid. Therefore, PARP might be the key for the ultimate apoptotic death of colon cancer cells induced by asiatic acid. Taken together, the results support the idea that the mitochondrial pathway plays an important role in apoptosis induced by asiatic acid in SW480 cells. Consistent with our findings, another study, asiatic acid induced dose- and time-dependent cell death in U-87MG human glioblastoma via decreased mitochondrial membrane potential, and activation of caspase-9 and caspase-3.

Asiatic acid induces not only apoptosis but also cell cycle S-G2/M arrest in human breast cancer cells. However, presently, cell cycle did not change appreciably except concerning apoptosis. Cho et al. found that asiatic acid induces both apoptosis and necrosis in U-87MG cells, but mainly induces apoptosis in colon cancer RKO cells. Bunpo et al. confirmed that asiatic acid inhibits growth of HT-29 colon cancer cells by affecting Bcl-2 and Bcl-xl, with both arrest in S-G2/M arrest in human breast cancer cells, but mainly induces apoptosis in colon cancer RKO cells. The results are consistent with the suggestion that asiatic acid shows discrepant mechanisms in different cells.

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REFERENCES