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ORIGINAL

Inhibitory effects of asiatic acid and CPT-11 on growth of HT-29 cells

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Abstract : Asiatic acid is a pentacyclic triterpene contained in medicinal plants. The cytotoxic effect of this compound and its augmentative effect on the anticancer drug irinotecan hydrochloride (CPT-11) were investigated in the human colon adenocarcinoma cell line HT-29. Asiatic acid dose-dependently showed cytotoxicity in HT-29 cells. DNA fragmentation, annexin-positive apoptotic cells, and caspase-3 activation were observed in a dose-dependent manner. A caspase-3 inhibitor suppressed the DNA ladder formation in a concentration-dependent manner. Bcl-2 and Bcl-x_L proteins were decreased by asiatic acid treatment. These results indicate that asiatic acid induced apoptosis in HT-29 cells via caspase-3 activation. Cytotoxic effects of combined treatment with CPT-11 and asiatic acid on HT-29 cells were further examined. Simultaneous treatment or sequential exposure first to asiatic acid and then to CPT-11 showed an additive effect. Synergism was observed when cells were first exposed to CPT-11 and then to asiatic acid. These results suggest that asiatic acid can be used as an agent for increasing sensitivity of colon cancer cells to treatment with CPT-11 or as an agent for reducing adverse effects of CPT-11. J. Med. Invest. 52 : 65-73, February, 2005

Keywords : *asiatic acid, apoptosis, CPT-11, combination, cytotoxicity*

INTRODUCTION

Asiatic acid is an active principle in *Centella asiatica* Linn., a medicinal plant. *C. asiatica* has been shown to inhibit the proliferation of transformed cell lines and to retard the development of solid and ascites tumors (1). We have reported that *C. asiatica* extract inhibited the formation of azoxymethane (AOM)-induced aberrant crypt foci (ACF) and AOM-induced tumorigenesis in the rat colon (2). Asiatic acid has a common structure of pentacyclic triterpenes and belongs to the amyrin ursolic acid group. Ursolic acid is widely distributed in medicinal herbs and edible plants (3, 4) and has been shown to exhibit growth inhibition properties against many human cancer cell lines (5-8). Lee *et al*. (9) reported that asiatic acid induces apoptosis in HepG2 human hepatoma cells. However, its activity for inducing apoptosis in various cancer cell lines has not been examined.

Apoptosis has recently become a subject of much interest in cancer chemotherapy. Bcl-2 family proteins are involved in the regulation of apoptosis either as death antagonists or death agonists (10-12). These antiapoptotic proteins act at upstream processes of activation of apoptotic proteases such as caspase-3 by preventing apoptotic signaling in cells (12, 13). While many tumor cells overexpress antiapoptotic proteins Bcl-2 and Bcl-x_L to become resistant to chemotherapy and radiotherapy (14, 15), some triterpenes have been reported to affect the level of expression of bcl-2 (16, 17).

Irinotecan hydrochloride (CPT-11), a water-soluble derivative of camptothecin (18), presents a wide spectrum of antitumor activity through inhibition of DNA

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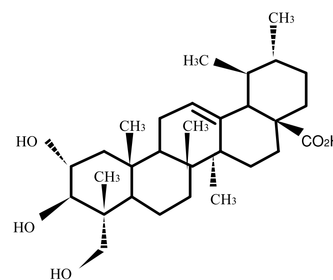
topoisomerase I. CPT-11 prevents DNA-religation reaction, resulting in DNA doublestrand breaks and eventually leading to apoptosis (19). It has shown cytotoxic activity in several malignant tumors, including cervical, breast, lung, ovarian, pancreatic, renal, colon and oesophageal cancers, leukemia and lymphoma (20-30). However, leukopenia and diarrhea are two major side effects in patients receiving CPT-11, often accompanied by cramping, flushing, and sweating. Grade 3-4 leukopenia and diarrhea were noted in several studies (26, 27, 31). Weekly chemotherapy regimens incorporating multiple drugs into one regimen have been developped to obtain the maximum antitumor effect with reduced adverse effects of the drugs (32). Recently, the antitumor effect of combinational therapy using an anticancer drug and a phytochemical has been studied (33-35). We previously reported that betulinic acid, a pentacyclic triterpene isolated from medicinal plants, augments the cytotoxic effect of vincristine on B16F10 melanoma cells (36). Since asiatic acid has been reported to induce apoptosis in hepatoma cells (9) and has a common structure of triterpenic acid, we expected that combinational treatment with CPT-11 and asiatic acid would show additive or synergistic cytotoxicity for human colon tumor cells.

In this study, we found that asiatic acid induced apoptotic cell death via caspase-3 activation. Furthermore, we evaluated the effectiveness of combinational treatment with CPT-11 and asiatic acid for HT-29 human colon cancer cells by isobologram analysis (37, 38).

A.



B.

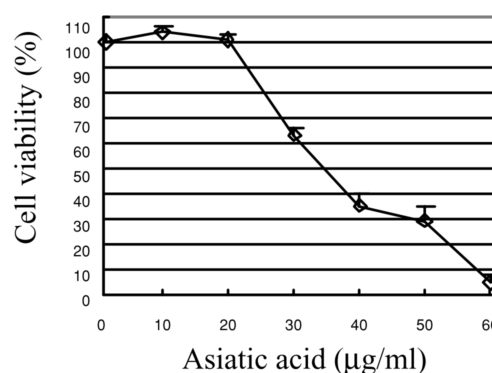


Fig 1. (A) Structure of asiatic acid (2, 3, 23-trihydroxyurs-12-en-28-oic acid). (B) Cytotoxicity of asiatic acid for HT-29 cells. Cells (1×10^4 cells/well) were exposed to various concentrations of asiatic acid for 24 h. Viable cell numbers were determined by using the MTS assay as described in MATERIALS AND METHODS. Each value is % of viable cell number of the solvent control and expressed as the mean ± SD calculated from 3 wells.

MATERIALS AND METHODS

Chemicals

Asiatic acid (Fig.1A) was purchased from Funakoshi Co., Ltd. Tokyo, Japan. CPT-11(irinotecan hydrochloride trihydrate) was purchased from Toronto Research Chemicals Inc., North York, ON, Canada. Other reagents were reagent grade or higher and obtained from Wako Pure Chemical Industries (Osaka, Japan).

Cell lines and culture conditions

Cells of the human colonic adenocarcinoma cell line HT-29(American Type Culture Collection, Rockville, USA) were grown at 37°C in a fully humidified atmosphere containing 5% CO_2. HT-29 cells were cultured in McCoy's 5A medium (ICN Biomedical, Ohio, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, GIBCO-BRL, Grand Island, NY), 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate. The number of viable cells was determined using a hemocytometer based upon their exclusion of 0.2% trypan blue dye.

Cytotoxicity

Cells were plated at 1×10^4 cells/well in 96-well culture plates, cultured for 24 h, and then treated with asiatic acid and/or CPT-11. Asiatic acid at final concentrations ranging from 10 to 60 μg/ml and CPT-11, from 10 to 200 μM, were added to the cultures in triplicate in a final volume of 100 μl. For combination studies, three different schedules of exposure were tested: asiatic acid and CPT-11 simultaneously for 24 h, CPT-11 for 24 h and then asiatic acid for 24 h, and asiatic acid for 24 h and then CPT-11 for 24 h. After drug exposure, the media in the control and drug-containing wells were removed and 100 μl of fresh media were added. Then 20 μl of 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2 H-tetrazolium, inner salt (MTS) (Promega, Corp., Madison, WI, USA) solution was added to each well according to the manufacturer's

instructions. The solutions were incubated for a certain time, and the absorbance was then measured at 490 and 630 nm. The results are calculated as follows : relative viability (%)=[(experimental absorbance−background absorbance)÷(absorbance of untreated controls −background absorbance)]×100.

DNA fragmentation analysis

DNA fragmentation in cells treated with asiatic acid was analyzed by the procedure of Ohyama (39). Briefly, 1×10^6 cells were lysed in 100 μl of chilled lysis buffer containing 10 mM Tris-HCl (pH7.4), 150 mM NaCl, 2.5 mM EDTA, and 1% Triton X-100. The samples were held on ice for 15 min, and then 2 μl of RNase A (1 mg/ml, Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 2 μl of RNase T1 (3,340 units/ml, Wako) were added and the mixtures were incubated at 37°C for 1h. The lysed cells were treated with 2 μl of proteinase K (Wako) at 50°C for 30 min. DNA was precipitated with isopropyl alcohol and dried by a speed vacuum concentrator (Tomy Seiko, Tokyo, Japan). The pellet was dissolved in Tris-EDTA buffer (pH 7.4). DNA (2-5 μg/lane) was electrophoresed in 2% agarose gel using Tris-borate EDTA buffer (pH 7.4) with a voltage of 100 V for 45 min, and DNA bands were stained with 0.5 μg/ml ethidium bromide and visualized under a UV transilluminator. If necessary, a caspase-3 inhibitor, Z-Asp-Glu-Val-Asp-FMK (Z : benzyloxycarbonyl, FMK : fluoromethylketone ; MBL Medical & Biological Laboratories Co., Ltd, Nagoya, Japan), was added at a concentration of 0.1-30 μM.

Assessment of cell death using flow cytometry

Cells(1×10^6) were seeded onto 6-well plates. After treatment with asiatic acid, cells were harvested and resuspended in 100 μl of a reaction mixture containing annexin V-FITC and propidium iodide (PI) according to the instructions of the manufacturer (R & D Systems, Minneapolis, MN). Cells binding annexin V but excluding PI were judged to be early apoptotic cells, whereas cells binding annexin V and accumulating PI were judged to be late apoptotic cells. In all experiments, fluorescence was determined from the combined collection of floating and attached cells by a flow cytometer (Coulter Epics XL-MCL, Beckman Coulter, Tokyo, Japan).

Western blot analysis

Whole cell lysates were prepared as described previously (40). Briefly, cells were harvested by centrifugation at 1,000 g for 5 min at 4°C. The cell pellets (3×10^6 cells) were washed once with ice-cold PBS and resuspended with 100 μl of the chilled lysis buffer containing 20 μg/ml leupeptin, 20 μg/ml aprotinin and 0.2 mM phenylmethylsulfonylfluoride (PMSF). Cells were disrupted by passing 10 times through a G 27 needle. After sequential centrifugation at 750 g for 5 min and at 15,000 g for 15 min at 4°C, the supernatants were divided into aliquots and stored at −20°C. Protein concentration was determined using a Coomassie protein assay kit (Pierce, Rockford, USA) according to the manufacturer's instructions. Samples were subjected to 12% SDS-polyacrylamide gel (Wako) electrophoresis with 200 V for 35 min and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA) with 20 V for 40 min. The membranes were blocked in TTBS (Tween 20-Tris-buffered saline) containing 2% bovine serum albumin (BSA) for 1 h and probed overnight with a primary antibody mouse anti-Bcl-2 and mouse anti-Bcl-x_L[1 : 1,000 ; BD Transduction, Japan] or with mouse anti-β-actin [1 : 10,000 ; Sigma Chemical Co., St. Louis, MO]) at 4°C. Primary antibody binding was detected with a goat anti-mouse IgG conjugated with alkaline phosphatase (1 : 2,000 ; Sigma) and visualized by an enhanced chemiluminescence method using disodium 3-(4-methoxyspiro {1,2-dioxetane, 3,2'-(5'-chloro) tricyclo[3.3.1.1^{3,7}] decan}-4-yl)phenyl phosphate (CSPD)(Boehringer, Manheim Germany).

Assay of caspase-3 activity

The activity of caspase-3 in HT-29 cells was measured using apopain substrate (Sigma) according to the user's manual (BD Biosciences Clontech Co., Palo Alto, CA). Cells(1×10^6) were plated for 24 h and exposed to asiatic acid for a further 12 h. Cells were collected by centrifugation, resuspended in 50 μl of the chilled lysis buffer as described above, and held on ice for 15 min. Fifty μl of the reaction buffer containing 1 μl of 1 M DTT and 5 μl of 1 mM acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC) was added to the cell lysate, and the mixture was incubated at 37°C for 1h. The fluorescence emitted at 450 nm (λ_{ex}=365 nm) was measured with a microplate reader spectrofluorometer (MTP-32 microplate reader, Corona Electric, Japan).

Drug interaction analysis

Additive or synergistic interaction between asiatic acid and CPT-11 was determined by using isobologram analysis as described in detail previously (37, 38). The type of interaction between asiatic acid and CPT-11 was evaluated by comparing the cytotoxic effects obtained after simultaneous or sequential exposures to the drugs with the ones observed after exposure to asiatic acid or CPT-11 alone. Interaction indices were

calculated by the following equation : interaction index= asiatic acid c/asitic acid e+CPT-11 c/CPT-11 e, where asiatic acid e and CPT-11 e are concentrations of asiatic acid and CPT-11 that inhibit 50, 60, 70% of proliferation when used alone, and asiatic acid c and CPT-11 c are concentrations of asiatic acid and CPT-11 that produce the same effect when used in combination. According to this method, an interaction index of less than 1.0 indicates synergistic interaction between two drugs, an interaction index of more than 1.0 indicates antagonism, and an index of 1.0 indicates additive interaction.

RESULTS

Cytotoxicity of asiatic acid

The viability of HT-29 cells exposed to asiatic acid for 24 h decreased in a dose-dependent manner to 4.8% of the control level at a dose of 60 μg/ml (Fig.1B). The concentration of 50% inhibition was 37.0±1.32 μg/ml. This result indicates that asiatic acid is cytotoxic to HT-29 cells.

DNA fragmentation

DNA ladder formation was observed in HT-29 cells after 24 h of incubation with asiatic acid at concentrations of 30, 40, 50 and 60 μg/ml in a dose-dependent manner (Fig.2A). It was observed in parallel with growth inhibition. DNA ladder formation caused by treatment with 50 μg/ml of asiatic acid for 24 h was suppressed by addition of the caspase-3 inhibitor Z-Asp-Glu-Val-Asp-FMK dose-dependently (Fig.2B).

Detection of early and late apoptotic cells by flow cytometric analysis

Early apoptotic cells appeared in a dose-dependent manner after treatment with 12.5 and 25 μg/ml of asiatic acid for 24 h(10.3 and 33.0%, respectively) (Fig.3). Late apoptotic cells accounted for 8.6 and 95.4% of total cells after treatment with 25 and 50 μg/ml of asiatic acid for 24 h, respectively.

Bcl-2 and Bcl-x_L expression in HT-29 cells

To elucidate involvement of Bcl-2 and Bcl-x_L proteins in the asiatic acid-induced apoptosis in HT-29 cells, the levels of these proteins were analyzed by Western blotting (Fig.4). When HT-29 cells were exposed to 50 μg/ml of

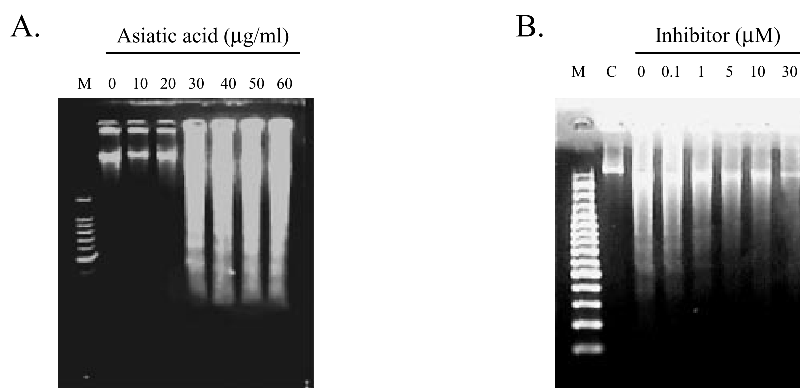


Fig 2. DNA fragmentation in HT-29 cells treated with asiatic acid. (A) Cells (1×10^6) were cultured in the absence or presence of 10-60 μg/ml of asiatic acid for 24 h. (B) HT-29 cells were treated with 0.1-30 μM Z-Asp-Glu-Val-Asp-FMK before adding 50 μg/ml of asiatic acid. DNA was then extracted from the cells and analyzed by 2% agarose gel electrophoresis. M, DNA size markers (100-bp ladders).

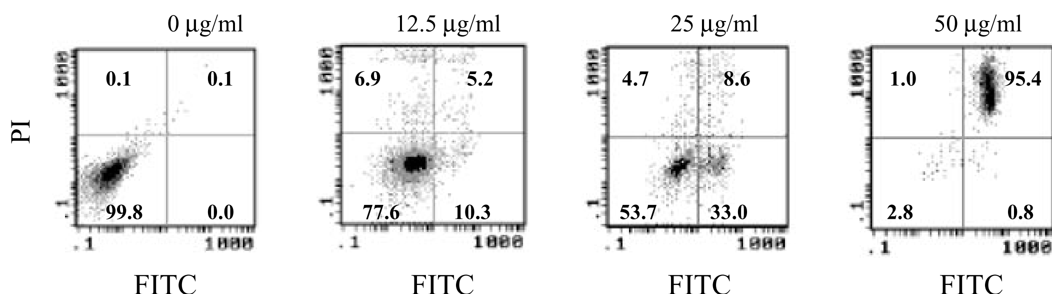


Fig 3. Flow cytometric analysis of apoptosis in HT-29 cells exposed to asiatic acid. Cells were treated with various concentrations of asiatic acid for 24 h and then stained with annexin V-FITC and PI. Values in the quadrants indicate percentages in the total cells.

asiatic acid for 6, 12 and 24 h, the levels of Bcl-2 and Bcl-x_L proteins decreased time-dependently. After 24 h, the levels of Bcl-2 and Bcl-x_L proteins standardized with the level of β-actin reached 0% and 26.3% compared with those at 0 h. These results suggest that down-regulation of the anti-apoptotic proteins Bcl-2 and Bcl-x_L may correlate with apoptosis in HT-29 cells.

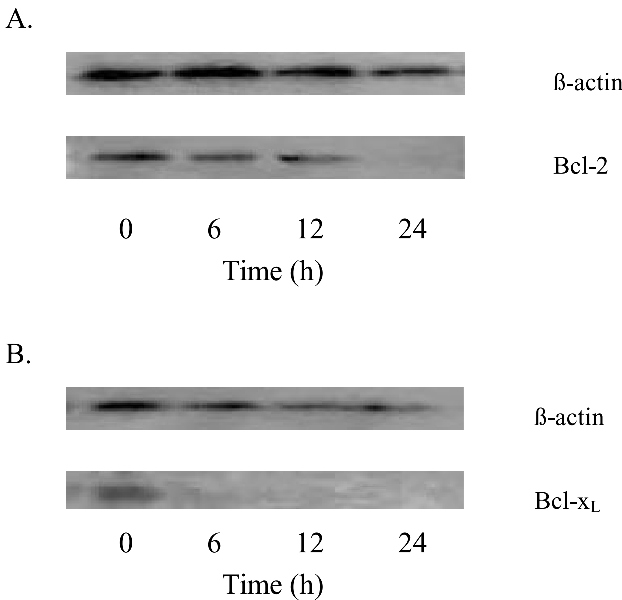


Fig 4. Western blot analysis. The expression levels of Bcl-2 (A) and Bcl-x_L(B) proteins in HT-29 cells after treatment with 50 μg/ml of asiatic acid were determined by Western blot analysis. Twenty μg of protein was loaded in each lane in 12% SDS-polyacrylamide gel electrophoresis. The protein recognized by each antibody is indicated on the side. β-actin was detected as an internal standard.

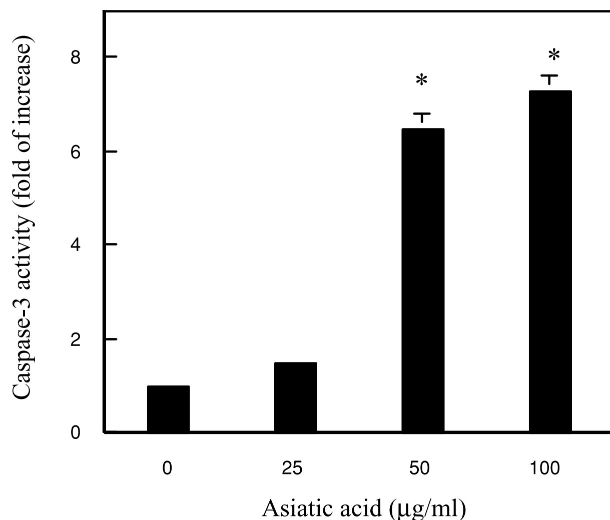


Fig 5. Activation of caspase-3 in HT-29 cells treated with asiatic acid. HT-29 cells (1×10^6) were incubated with 25, 50 and 100 μg/ml of asiatic acid for 12 h. Values are means±SD. Bars with * are significantly different from the 0 μg/ml treatment group at p<0.0001.

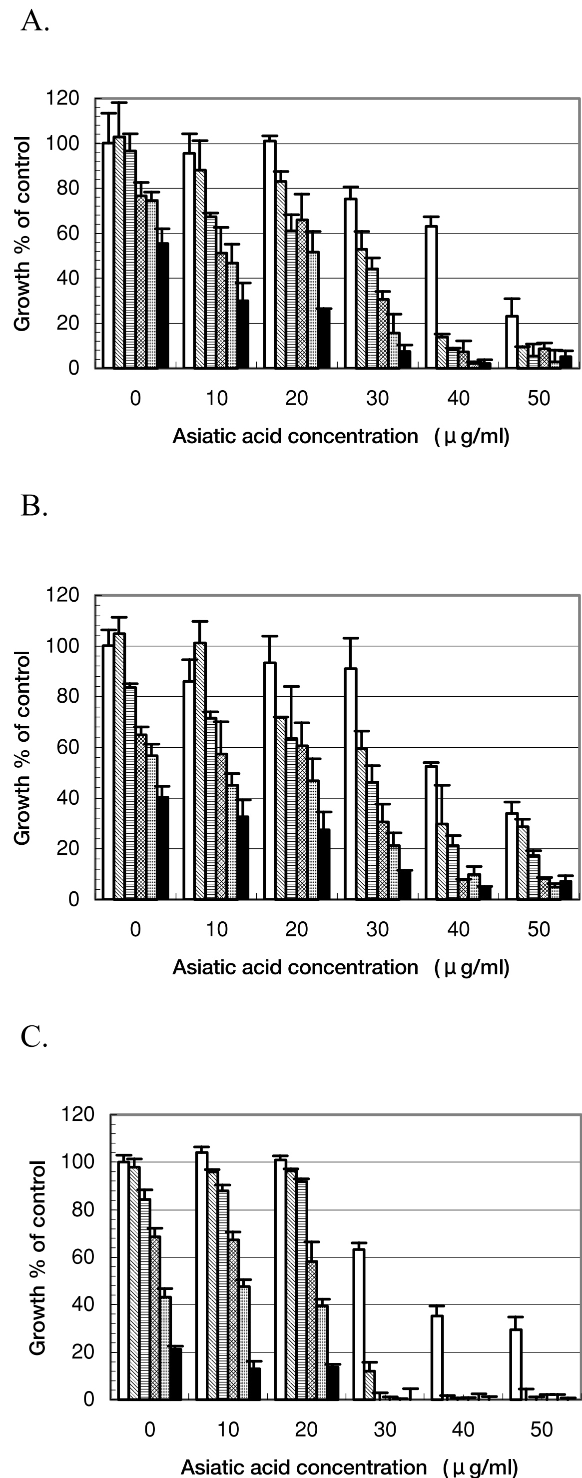


Fig 6. Effect of combinational treatment of asiatic acid and CPT-11 on the growth of HT-29 cells. (A) The cells were treated with asiatic acid and CPT-11 simultaneously. (B) The cells were treated with asiatic acid for 24 h and then with CPT-11 for a further 24 h. (C) The cells were treated with CPT-11 for 24 h and then with asiatic acid for a further 24 h. Concentrations of CPT-11 were 0 μM (open box), 20 μM (hatched box), 40 μM (crossed box), 80 μM (latticed box), 100 μM (dotted box) and 150 μM (closed box). Cytotoxicity was evaluated using the MTS assay. Data are expressed as means±SD of three independent experiments.

Activation of caspase-3 in HT-29 cells

To investigate the activation of caspase-3 during apoptosis, activity of caspase-3 was measured by fluorometric analysis using Ac-DEVD-AMC, a caspase-3-specific synthetic substrate. Fig.5 shows significant activation of caspase-3 in a dose-dependent manner in HT-29 cells ($p<0.0001$). Caspase-3 activity had increased to 6.3 fold and 7.1 fold of the solvent control ($P<0.0001$) at 12 h after asiatic acid treatment at concentrations of 50 and 100 μg/ml, respectively.

Cytotoxicity of the asiatic acid/CPT-11combination

CPT-11 alone inhibited the growth of HT-29 cells in a dose-dependent manner (Fig.6). Asiatic acid alone also inhibited the growth of HT-29 cells in a dose-dependent manner (Fig.6). Since asiatic acid and CPT-11 showed cytotoxicity after 24 h at the concentrations of 10 to 50 μg/ml (Fig.1) and 20 to 150 μM (Fig.6), respectively, combinational treatment was done at these concentrations for 24 h. When the cells were treated simultaneously with asiatic acid and CPT-11, interaction indices at 50, 60 and 70% inhibition of proliferation were 0.98 ± 0.17, 0.92 ± 0.08 and 0.93 ± 0.09, respectively, indicating an additive effect (Fig.6A). When cells were first exposed to asiatic acid and then treated with CPT-11, interaction indices were 0.99 ± 0.10, 0.93 ± 0.15 and 0.88 ± 0.17, respectively, indicating an additive effect (Fig.6B). When cells were sequentially exposed to CPT-11 and then asiatic acid, interaction indices at 50, 60 and 70% inhibition of proliferation were 0.87 ± 0.08, 0.81 ± 0.05 and 0.76 ± 0.03 (Fig.6 C). A weak synergism was observed in this treatment.

DISCUSSION

Asiatic acid is an active principle in *C. asiatica*. Crude extracts of this medicinal plant have shown chemopreventive effects in *in vivo* tumor models (1, 2). In an *in vitro* experiment, asiatic acid induced apoptosis in HepG2 human hepatoma cells (9). In this study, we examined asiatic acid-induced apoptosis in human colon tumor-derived cells, HT-29 cells. Asiatic acid dose-dependently showed cytotoxicity in HT-29 cells (Fig. 1B). After asiatic acid treatment, DNA ladder formation was observed (Fig.2A) and flow cytometric analysis showed that annexin-positive cells increased dose-dependently (Fig.3). Caspase-3, one of the effector proteases in an apoptosis process (41), was activated by asiatic acid (Fig.5). In the presence of the caspase-3 inhibitor Z-Asp-Glu-Val-Asp-FMK, DNA fragmentation triggered by asiatic acid was inhibited (Fig.2B). These results indicate that asiatic acid induced apoptosis through activation of caspase-3, which cleaves DNA fragmentation factor 45 (DFF45) in the DFF45/DFF 40 complex (42) and produces active DFF40 to trigger chromosomal DNA fragmentation.

Bcl-2 is an antiapoptotic protein, predominantly present in the outer mitochondrial membrane, the endoplasmic reticulum membrane and the nuclear membrane(13). Bcl-2 has been shown to inhibit cytochrome c release from mitochondria into the cytosol by inhibiting insertion of the proapoptotic protein Bax in the mitochondria or by directly or indirectly inhibiting Bax-channel activity (13). Bcl-x_L is also an antiapoptotic protein present in the cytosol, and it works to close the channel (13, 43). Many tumor cells overexpress these antiapoptotic proteins and become resistant to chemotherapy and radiotherapy (14,15). Ohmori *et al.* (44) reported that bcl-2 can modulate the cytotoxicity of some anti-cancer agents such as CPT-11 and mitomycin C by inhibiting the process of apoptosis. In the present study, the levels of Bcl-2 and Bcl-x_L were decreased by treatment with asiatic acid, suggesting that down-regulation of Bcl-2 and Bcl-x_L in response to asiatic acid may cause apoptosis. Since HT-29 cells carry high levels of non-functional p53(45), asiatic acid-induced apoptosis in these cells may not be mediated by activation of p53 but triggered by decrease in levels of Bcl-2 and Bcl-x_L after asiatic acid treatment.

Systemic chemotherapy of colorectal cancer using new agents that target specific molecular processes of cell proliferation, vascularization, metastasis and apoptosis inhibition have been developed (32). CPT-11 is a topoisomerase I inhibitor (18) and has been clinically applied for treatment of patients with colorectal cancer that is refractory to treatment with fluorouracil (26, 27). Sensitivity of tumor cells to a topoisomerase inhibitor depends on topoisomerase I activity (19), tumor-associated deficiency of p53(46), and the easiness of apoptosis induction (47, 48). Combination with apotosis-inducing agents would enhance the chemotherapeutic response of colorectal cancer treated with topoisomerase I inhibitors. In this study, we examined effects of combinational treatment with CPT-11 and asiatic acid on cytotoxicity for HT-29 cells according to three kinds of protocol : 1)simultaneous treatment, 2)first exposed to asiatic acid and then treated with CPT-11, 3) first exposed to CPT-11 and then treated with asiatic acid. Simultaneous treatment and sequential treatment in which cells were first treated with asiatic acid and then with CPT-11 showed an additive cytotoxic effect. Since asiatic acid is a derivative of ursolic acid, which has been reported to block cell cycle progression in the

G1 phase (49), it is possible that a part of the HT-29 cell population treated with asiatic acid is blocked in G1 phase and can no longer respond to CPT-11. The remaining part of the cell population might enter S phase and respond to CPT-11. On the other hand, synergism was observed when cells were first exposed to CPT-11 and then treated with asiatic acid. In CPT-11-pretreated cells, asiatic acid-mediated decrease in the antiapoptotic proteins Bcl-2 and Bcl-x_L might enhance the apoptotic process, resulting in synergistic cytotoxicity. Hayward *et al.* (48) reported that antisense-mediated Bcl-x_L knockdown enhances the response to topoisomerase I inhibition in the colorectal cancer cell line HCT 116. Although cytotoxic intensity of the combinational treatment was dependent on the order of the treatment, asiatic acid could possibly be used to enhance the tumor cell-killing effect of the anticancer drug CPT-11.

Combinational treatment with CPT-11 and asiatic acid revealed additive or synergistic cytotoxicity in HT-29 cells. The total triterpenic fraction of *C. asiatica*, in which asiatic acid is one of main constituents, has been used for treatment of venous hypertension in clinical studies at a dose of 30 or 60 mg/day for 10 weeks or 4 months (50, 51). Taken together, the results of this study suggest that asiatic acid enhances the sensitivity of a tumor to anticancer drugs and reduces the adverse effects of chemotherapy. The mechanism of asiatic acid-induced apoptosis in colon cancer cells should be clarified for introducing this compound to clinical use.

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