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Supernatant of bacterial fermented soybean induces apoptosis of human hepatocellular carcinoma Hep 3B cells via activation of caspase 8 and mitochondria

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Abstract

SC-1, the aqueous phase of soybean fermentation products by bacteria (*Bacillus subtilis* and *Bacillus brevis*), significantly inhibited the growth and clonogenesity of human hepatocellular (Hep 3B), mouse hepatocellular (ML-1), and human colorectal (HCT 116 and HT-29) carcinoma cells. Cytotoxicity of SC-1 in Hep 3B cells was through the process of apoptosis characterizing by increase in cell population of sub-G_1 phase, fragmentation of DNA, and change of nuclear morphology. Treatment of Hep 3B cells with SC-1 activated caspase 8 and caspase 3. Elevation of nuclear DNA fragmentation factor 40 (DFF40) and cleavage form of poly(ADP-ribose) polymerase (PARP) were also observed. SC-1 also activated intrinsic pathway via increase of pro-apoptotic (tBid, Bak and Bax) and decrease of anti-apoptotic (Bcl-2 and Bcl-x_L) proteins on mitochondria, disruption of mitochondrial membrane potential, release of cytochrome *c* and Smac (second mitochondria-derived activator of caspase/direct IAP binding protein with low PI) from mitochondria, and activation of caspase 9. Inhibition on protein expression of Ku70 in cytosol and cyclooxygenase (COX)-2, but not COX-1, in whole cell lystes were revealed in SC-1-treated Hep 3B cells. These results suggest caspase 8, Ku70 and mitochondria are involved in the antitumor mechanism of SC-1 in Hep 3B cells.
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Keywords: Fermented soybean; Apoptosis; Mitochondria; Caspases; Cyclooxygenase

Abbreviations: Apaf-1, Apoptotic protease activating factor-1; ATCC, American type culture collection; *B. brevis*, *Bacillus brevis*; *B. subtilis*, *Bacillus subtilis*; COX, Cyclooxygenase; DFF, DNA fragmentation factor; DMEM, Dulbecco's modified Eagle medium; DTT, Dithiothreitol; EDTA, Ethylenediamine-tetraacetic acid; EGTA, Ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N'*,*N'*-tetraacetic acid; FBS, Fetal bovine serum; HBS, HEPES buffer solution; HBV, Hepatitis B virus; HCC, Hepatocellular carcinoma; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid; HRP, Horseradish peroxidase; MTT, 3-[4,5]-dimethylthiazol-2-yl-2,5-diphenyltetra-zolium bromide; PARP, Poly(ADP-ribose)polymerase; PBS, Phosphate buffered saline; PI, Propidium iodide; PMSF, Henylmethysufonyl fluoride; SC-1, The aqueous phase of soybean fermentation products by bacteria *B. subtilis* and *B. brevis*; SDS, Sodium dodecylsulphate; SDS-PAGE, SDS-polyacrylamide-gel electrophoresis; Smac, Second mitochondria-derived activator of caspase/direct IAP binding protein with low PI; tBid, Truncated Bid; TBST, Tris buffer solution with tween; XIAP, X-linked apoptosis-inhibiting protein; $\Delta\psi_m$, Mitochondrial membrane potential.

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1. Introduction

Soybean products such as tofu, soy milk (*tonyu*), miso and soy sauce are Asian traditional food. Consumption of soy foods, the predominant source of isoflavones, has been reported to reduce breast, colon and prostate cancer risk (Birt et al., 2001). Phosphatidyl inositol, saponins and sphingolipids in soybeans also exhibit tumor prevention properties in experimental animal model (Fournier et al., 1998). Recently, soy fermentation products and their ingredients are found to act as chemoprevention agents (Yang et al., 1997; Ohta et al., 2000; Chang et al., 2002). To determine the antitumor effect of a Taiwan traditional remedy, the supernatant of fermented soybean products by *Bacillus subtilis* (*B. subtilis*) and *Bacillus brevis* (*B. brevis*) was used for evaluation of apoptotic efficacy.

Apoptosis (the programmed cell death) plays a pivotal role during development, homeostasis and immune response in multicellular organisms (Jacobson et al., 1997; Earnshaw et al., 1999). Cells fail to die of apoptosis contribute to oncogenesis, autoimmunity and degenerative disorders (Williams, 1991; Vaux and Korsmeyer, 1999). Stimuli including radiation, tumor necrosis factor and certain chemotherapeutic agents trigger apoptosis of tumor cells (Amarante-Mendes et al., 1998; Hu and Kavanagh, 2003). Two major pathways have been reported. In receptor-mediated pathway, interaction with death receptors causes the activation of caspase 8, caspase 3 and their downstream regulators (Nothwehr and Martinou, 2003; Belka et al., 2004). In mitochondrial pathway, increase in pro-apoptotic proteins of Bcl-2 family breakdowns mitochondrial membrane potential ($\Delta\psi_m$), causes the release of cytochrome *c* and results in the activation of caspase 9 and caspase 3 (Danial and Korsmeyer, 2004). These two mechanisms link at the activation of caspase 8 which cleaves Bid and results in the release of apoptogenic proteins from mitochondria (Roth and Reed, 2002).

Human hepatocellular carcinoma (HCC) is the fifth most frequent cancer worldwide, and is the third most common cause of cancer-related death (Llovet et al., 2003). In the present study, the anticancer efficacy of SC-1 was determined on hepatitis B virus (HBV) related HCC cells (Hep 3B) since chronic HBV infection further increases the relative risk of developing HCC to a 100-fold compared with non-infected individuals (Beasley et al., 1981). Our results indicate that SC-1, the aqueous phase of soybean fermentation products by bacteria (*B. subtilis* and *B. brevis*), is a potent anticancer agent. Induction of apoptosis via both caspase 8 and mitochondria is observed in SC-1 treated Hep 3B cells.

2. Materials and methods

2.1. Reagents

Most of the chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated. Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from GIBCO BRL (Grand Island, NY). Penicillin and streptomycin were purchased form Y. F. Chemical Corp. (Taipei, Taiwan). Glycine and protein assay reagents were obtained from Bio-Rad Laboratories (Hercules, CA). Phosphate buffered saline (PBS), $Na_2HPO_4 \cdot 12H_2O$, $NaH_2PO_4 \cdot 2H_2O$, $Na_4P_2O_7 \cdot 10H_2O$, NaCl, methanol and ethanol were purchased from Wako (Osaka, Japan). Triton X-100, RNase A, ethylenediamine-tetra-acetic acid (EDTA), NaF, sodium dodecylsulphate (SDS) and NaN_3 were purchased from Merck (Darmstadt, Germany). Other reagents were obtained from the following sources: dithiothreitol (DTT, MDBio, Taipei, Taiwan); proteinase K (BD Biosciences Clontech, Palo Alto, CA); Tris–HCl (American Biorganics, Niagara Falls, NY); Tween-20 (SHOWA Chemical, Tokyo, Japan).

Antibodies to various proteins were obtained from the following sources: anti-caspase 3 mouse monoclonal and anti-second mitochondria-derived activator of caspase/direct IAP binding protein with low PI (Smac) rabbit polyclonal antibodies were purchased from IMGENEX (San Diego, CA); anti-X-linked apoptosis-inhibiting protein (XIAP), anti-cytochrome *c* mouse monoclonal antibodies and anti-Bid, anti-poly(ADP-ribose) polymerase (PARP) rabbit polyclonal antibodies were purchased from BD Pharmingen (San Diego, CA); anti-caspase 9 mouse monoclonal antibody was purchased from Upstate (Lake Placid, NY); anti-caspase 8 mouse monoclonal antibody was purchased from Cell Signaling (Beverly, MA); anti-Ku70, anti-Bcl-2, anti-Bax, anti-Bcl-x_L, anti-cyclooxygenase (COX)-1 and anti-COX-2 mouse monoclonal antibodies, anti-Bak, anti-DNA fragmentation factor (DFF) 45 and anti-DFF40 rabbit polyclonal antibodies, goat anti-mouse and donkey anti-goat conjugated horseradish peroxidase (HRP) secondary antibodies were purchased from Santa Cruz Biotech (Santa Cruz, CA); goat anti-rabbit conjugated HRP secondary antibody was purchased from Amersham Pharmacia Biotech (Piscataway, NJ); anti-tBid rabbit polyclonal antibody was purchased from Biosource (Camarillo, CA); anti-β-actin was purchased from Sigma (St. Louis, MO).

2.2. Preparation of fermented soybean supernatant

Soybeans (1 kg) obtained from Dongshi Shiang, Chiayi, Taiwan were ground, boiled for 8 h and soaked in sterile water (10 l) for 10 days. After centrifugation at $1000 \times g$, the supernatant was cultured with *B. subtilis* (10^5 cells/ml) and *B. brevis* (10^5 cells/ml) for 1 month at 37 °C. Before the experiments, SC-1 was prepared by centrifuged this fermentation product at $15{,}000 \times g$ for 30 min, filtered through 0.22 μm filter (Corning, Corning, NY) to avoid the bacteria contamination, freeze-dried, and stored at −70 °C until use. All solutions were prepared in pyrogen-free glassware that was heated for 5 h before use.

2.3. Cell culture

Human colorectal carcinoma (HCT 116 and HT-29) and HCC (Hep 3B) cells were obtained from American Type Culture Collection (ATCC, Rockville, MD). Mouse hepatoma ML-1 cells (Chen et al., 1992) were obtained from Dr. Huan-Yao Lei (Department of Microbiology and Immunology, Medical College, National Cheng Kung University). Cells were grown in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 unit/ml penicillin and 100 μg/ml streptomycin. Cells were maintained at 37 °C in a 5% CO_2 humidified atmosphere.

2.4. Viability assay

The inhibition of cell viability was assessed by modified colorimetric assay (Mosmann, 1983). Cells (1.5×10^3 cells/well) were seeded in 96-well/plates (Nunc, Denmark) for 12 h. After attachment to plates, the supernatants in each well were replaced very carefully with 100 μl of fresh DMEM containing different concentrations of SC-1. The plates were then incubated for 5 days. Cell viability was determined by adding 10 μl of 3-[4,5]-dimethylthiazol-2-yl-2,5-diphenyltetra-zolium bromide (MTT) at a final concentration of 0.5 mg/ml in PBS to each well. The MTT was taken up and converted to a purple formazan. After 4 h of incubation, 100 μl of 10% SDS in 0.01 N HCl was added to each well to dissolve the formazan

for overnight. The absorbance of each well was measured in a microplate reader at 590 nm on a Multiscan photometer (MRX II, Dynatech, McLean, VA). IC_{50} was the concentration that achieved 50% cytotoxicity against culture cells.

2.5. Colony formation assay

Cytotoxicity was determined to evaluate the ability of tumor cells to form colonies with SC-1 treatment. The assay was performed using a two-layer agar system in 6-well/plates (Nunc, Denmark). As described previously (Wang et al., 2005), the base layer was 0.6% agar containing DMEM with 10% FBS, and the upper layer was 0.33% agar in DMEM supplemented with 10% FBS and contained tumor cells (6×10^4 cells/well) and various concentrations of SC-1. Colonies with diameter larger than 1 mm were counted 14 days later (Stewart et al., 1999).

2.6. Flow cytometry analysis for cell cycle distribution

The measurement was assessed by the reported method (Di Leonardo et al., 1994). As described previously (Lee et al., 2005), cells (2×10^5 cells/well) were seeded into 6-well/plates (Nunc, Denmark). After treated with different concentrations of SC-1 for 48 or 72 h, cells were trypsinized and resuspended in HEPES buffer solution (HBS). After centrifugation at $800 \times g$ for 10 min at 4 °C, cells were resuspended in 70% ethanol at 4 °C for overnight. The samples were washed and resuspended in HBS containing 40 μg/ml of propidium iodide (PI) and 100 mg/ml of RNase A in the dark for 30 min. Cell cycles (G_0/G_1, S and G_2/M) were determined by DNA histograms obtained by flow cytometry (Becton Dickinson Immunocytometry system). Results were analyzed with the Windows Multiple Document Interface software for Flow Cytometry (WinMDI 2.8, Scripps Research Institute, San Diego, CA).

2.7. DNA fragmentation assay

As described previously (Wang et al., 2005), SC-1 treated or untreated cells (1×10^6 cells/well) were incubated with lysis buffer containing 10 mM Tris–HCl [pH 7.6], 1 mM EDTA and 1% IGEPAL CA-630 (NP-40) at 37 °C for 20 min. Cell lysates were centrifuged and the supernatants were incubated with the solution that contained 0.5% of SDS and 4 mg/ml of RNase A at 56 °C for 2 h. After incubation, 40 μl of proteinase K (20 mg/ml) was added to a final concentration of 1 mg/ml and the mixtures were incubated at 37 °C for overnight. DNA was precipitated with 0.1 volume of ammonium acetate (10 M) and 2.5 volume of ethanol at −20 °C for overnight. Pellets were obtained after centrifugation at $13{,}000 \times g$ for 15 min. DNA samples were stained with ethidium bromide and separated on 1% agarose gel. The fragments of DNA were examined by exposing to UV light (Sandstrom and Buttke, 1993).

2.8. Determination of nuclear morphologic changes

Cells (1×10^5 cells/well) on 6-well/plates (Nunc, Denmark) were incubated without or with SC-1 for various time periods (0–72 h). After trypsinization and washing with PBS, cells were fixed with 4% paraformaldehyde in PBS for 5 min. Following washing three times with PBS, cells were spun on glass microscope slides and permeabilized in PBS containing 0.1% Triton X-100 and 0.05% Tween-20. Staining was carried out in the dark by incubating cells with 0.5 μg/ml of Hoechst 33258 in PBS at 37 °C for 30 min. Cells were washed three times and mounted with 3 μl of rapid mounting media (Merck, Darmstadt, Germany). Changes of nuclear morphology were visualized under fluorescence microscope (Leica DMRBE microscope).

2.9. Fractionation of cell proteins

Cells (5×10^5 cells/well) were treated without or with SC-1 for various time periods (0–72 h). The methods of protein extraction were performed as previously described (Feng and Lo, 1999; Watabe et al., 2000; Lee et al., 2005). Briefly, whole cells were lysed with 200 μl lysis buffer containing 10 mM Tris–HCl [pH 7.9], 0.15 M NaCl, 1% (w/v) Triton X-100, 5 mM EDTA, 10 mM NaF, 10 mM NaN_3, 5 mM $Na_2HPO_4 \cdot 12\,H_2O$, 5 mM $NaH_2PO_4 \cdot 2H_2O$, 5 mM $Na_4P_2O_7 \cdot 10H_2O$, and 1 tablet of complete protease inhibitor cocktail (Boehringer, Mannheim, Germany). Cell mixtures were centrifuged at $15{,}000 \times g$ for 10 min and the resulting supernatants were used as the whole cell lysates for immunoblotting.

Cytosolic and mitochondrial proteins were prepared as described previously (Earnshaw et al., 1999; Watabe et al., 2000). Briefly, cells (5×10^5 cells/well) were washed with PBS and harvested by centrifugation at $800 \times g$ for 10 min at 4 °C. The pellets were washed twice with ice-cold PBS and were then resuspended in TSE buffer (10 mM Tris, 0.25 M sucrose, 0.1 mM EDTA, pH 7.4). Cell suspensions were transferred to a Dounce homogenizer (Glas-Col, Terre Haute, IN) and broken with 10 strokes of a Teflon pestle. The homogenates were centrifuged at $750 \times g$ at 4 °C for 30 min. The supernatants were centrifuged at $12{,}000 \times g$ for 30 min at 4 °C. The lysed solutions were centrifuged at $100{,}000 \times g$ for 1 h and their resulting supernatants were used for cytosolic fractions. The obtaining pellets were incubated with 100 μl lysis buffer containing 10 mM Tris–HCl [pH 7.9], 0.15 M NaCl, 1% (w/v) Triton X-100, 5 mM EDTA, 10 mM NaF, 10 mM NaN_3, 5 mM $Na_2HPO_4 \cdot 12\,H_2O$, 5 mM $NaH_2PO_4 \cdot 2H_2O$, 5 mM $Na_4P_2O_7 \cdot 10H_2O$, and 1 tablet of complete protease inhibitor cocktail (Boehringer, Mannheim, Germany). The lysed solutions were used as mitochondrial fractions for immunoblotting.

Nuclear fractions were prepared as previous described (Feng and Lo, 1999). Cells (5×10^5 cells/well) were isolated by centrifugation, washed twice with ice-cold PBS, lysed in 400 μl of buffer A (10 mM HEPES [pH 7.9], 5 mM $MgCl_2$, 10 mM KC1, 3 mM Na_3VO_4, 10 mM NaF, 0.5 mM DTT, 0.5 mM phenylmethysufonyl fluoride (PMSF), 2 μg/ml leupeptin and 2 μg/ml pepstatin A), and incubated on ice for 20 min. After centrifugation at $11{,}000 \times g$ at 4 °C for 10 s, the pellets were resuspended in 60 μl of buffer B (20 mM HEPES [pH 7.9], 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, 25% glycerol, 3 mM Na_3VO_4, 10 mM NaF, 0.5 mM DTT, 0.5 mM PMSF, 2 μg/ml leupeptin and 2 μg/ml pepstatin A) and incubated for 15 min on ice with occasional mixing. Debris was removed by centrifugation at $12{,}000 \times g$ for 15 min at 4 °C. The obtained nuclear proteins were used for immunoblotting.

2.10. Immunoblotting

Protein contents of whole cell, cytosolic, mitochondria and nuclear fractions were determined by protein assay kit (Bio-Rad, Hercules, CA). All isolated proteins were stored at −80 °C before use. Proteins were resolved using 10–12% SDS polyacrylamide gel electrophoresis (PAGE) with running buffer (25 mM Tris, 192 mM glycine, 3.5 mM SDS, pH 8.3) and subsequently transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA) as described previously (Chendil et al., 2002). Membranes were blocked by incubating in TBST (20 mM Tris, 137 mM NaCl, 0.05% Tween-20, pH 7.4) containing 5% skim milk for 2 h at room temperature. Follow by probed the membrane with a appropriate first antibody, a secondary probe with HRP-labeled goat anti-mouse (1:5000), goat anti-rabbit (1:5000) or donkey anti-goat (1:5000) antibody was visualized by exposure to X-ray film (Kodak, PerkinElmer, Rochester, NY) after staining with chemiluminescence reagents (PerkinElmer, Boston, MA).

2.11. Determination of $\Delta\psi_m$ by confocal microscopy

$\Delta\psi_m$ was measured by using a laser scanning confocal microscope (Leica TCS-SP2, Germany) as described previously (Yang et al., 1997; Lee et al., 2005). Briefly, cells (1×10^5 cells/well) were plated in 6-well/plates (Nunc, Denmark) containing methanol-sterilized glass cover slips. After overnight incubation, cells were treated or untreated with various concentrations of tested agent for the indicated time periods and then stained with 5 μM rhodamine 123 at 37 °C for 30 min. After washing with PBS, cells were fixed with 4% paraformaldehyde for 15 min, mounted onto

poly-L-lysine coated glass microscope slides (Menzel-Glaser, Germany) and examined immediately. Samples were viewed in the dark with a Leica TCSNT laser scanning confocal imaging system coupled to a Leica DMRBE microscope, using a Leica 630 fluotar objective. The stained cells were excited with the 488-nm lines of a 25-mW laser. The laser was set to the optimum power that could produce a fluorescent signal. Rhodamine 123 fluorescence was visualized with a BF530/30 emission filter combination. Optical sections close to the middle of the cells were chosen for visual evaluation of experimental treatments.

3. Results

3.1. SC-1 inhibits the growth of tumor cells

To examine whether the supernatant of the fermented soybeans would alter the malignant proliferation, the inhibitory effects of SC-1 on the growth of tumor cells were determined by a modified MTT colorimetric assay. As shown in Table 1, the IC_{50} value (50% cell growth inhibitory concentration) for human HCC (Hep 3B), mouse hepatoma cells (ML-1) and human colorectal carcinoma (HCT 116 and HT-29) cells were 53.7, 58.7, 54.5 and 48.4 μg/ml of SC-1, respectively. A time (0–5 days) and dose (20–165 μg/ml) related inhibition of the cell growth by SC-1 was also observed (data not shown). To further analyze the ability of SC-1 treated tumor cells on anchorage-independent growth properties, the effect of SC-1 on clonogenic survival was evaluated by colony formation assay. After 14 days of incubation, SC-1 decreased the number of colonies to 50% at the concentrations of 47.7, 65.9, 75.2 and 55.2 μg/ml for Hep 3B, ML-1, HCT 116 and HT-29 cells, respectively (Table 1). The data demonstrate that these tumor cells are comparably sensitive to SC-1 on the suppression of cancerous growth and colony formation.

3.2. Induction of apoptosis by SC-1

Induction of apoptosis has been reported to be a potentially promising approach for cancer therapy (Green, 2000). Exhibition of apoptotic phenomena (cell cycle redistribution, DNA fragmentation, and chromatin condensation) represents the proceeding of apoptosis (Nicoletti et al., 1991; Danial and Korsmeyer, 2004). To examine the possible anti-proliferation mechanism of SC-1 in HCC cells, cell cycle distributions of Hep 3B cells cultured with SC-1 (0–662.5 μg/ml) for 48 or 72 h were analyzed by flow cytometry. After staining the DNA with PI, the percent of hypodipliod DNA content was determined. As shown in Fig. 1A, a dose-related accumulation of cells at sub-G_1 phase was observed. At the concentration of 0, 41.4, 82.8, 165.6, 331.23 and 662.5 μg/ml, SC-1 increased the percentage of cells at sub-G_1 phase from 0.7 to 1.5 (2.1-fold), 1.6 (2.3-fold), 2.7 (3.9-fold), 37.7 (53.9-fold) and 57.7% (82.4-fold) at 48 h, and from 1.2 to 3.0 (1.8-fold), 3.3 (2.8-fold), 5.1 (4.3-fold), 40.7 (33.9-fold) and 60.3% (50.3-fold) at 72 h (Fig. 1A). The data indicate that within the range of 0–662.5 μg/ml and over the incubation time of 0–72 h, SC-1 increases in the percentage of cells at the sub-G_1 phase in a dose- and time-related manner. For detection of DNA fragmentation, agarose gel electrophoresis was performed. After 72 h incubation, a dose-related (0–662.5 μg/ml) increase in DNA ladders was displayed (Fig. 1B). A time-related effect (24, 48 and 72 h of incubation time) of SC-1 on DNA fragmentation was also observed (data not shown). For nuclear morphology assay, Hep 3B cells were cultured with 165.6 μg/ml of SC-1 for various time periods (0–72 h). Chromatin condensation was visualized by staining cells with Hoechst 33258. As shown in Fig. 1C, control cells appeared to be morphologically normal with intact DNA. As incubation time increased, the number of cells with condensed chromatin increased especially at the time of 48 or 72 h. These results demonstrate that SC-1 induces apoptosis of Hep 3B cells *in vitro* in a SC-1 dose and/or incubation time-related manner.

Table 1
IC_{50} of cell growth and colony formation on human and mouse tumor cell lines

Cell line	Growth inhibition (μg/ml)	Clonogenic cell inhibition (μg/ml)
Hep 3B	53.7 ± 4.6[a,b]	47.7 ± 1.2
ML-1	58.7 ± 0.8	65.9 ± 6.6
HCT 116	54.5 ± 4.5	75.2 ± 9.4
HT-29	48.4 ± 2.8	55.2 ± 4.4

[a] Control cells were treated with DMEM. Experiments were repeated three times and each concentration of samples was conducted in eight replicates.
[b] The experimental data are expressed as mean ± SEM.

3.3. SC-1 activates caspases in Hep 3B cells

Apoptosis can be carried out by the activation of caspases in which caspase-2, -8 and -9 are initiator caspases and caspase-3 is classified into effecter caspases (Li and Yuan, 1999). To determine the signal pathway in SC-1 treated Hep 3B cells, cells were incubated with 165.6 μg/ml of SC-1 for 0–72 h. Whole cell lysates and nuclear proteins were obtained for immunoblotting. SC-1 increased the expression of activated cleavage form of caspase 8 at 12 h (Fig. 2A), and the activation of its downstream caspase 3 was also observed at 12 h (Fig. 2B). In contrast, activation of caspase 2 was not detected (data not shown). The substrates of activated caspase-3 include PARP (Chinnaiyan et al., 1995) and DFF45 (Enari et al., 1998). The present results exhibit that in the presence of SC-1 (165.6 μg/ml), nuclear full-length PARP (116 kDa) decreased and its cleavage form (89 kDa) increased at 24 h (Fig. 2C and E). SC-1 also significantly decreased total DFF45 and DFF35, and increased nuclear DFF40 expressions (7.1, 11.9 and 29.8-fold at 12, 24 and 72 h) (Fig. 2D and E). The data suggest that SC-1 may act through the initiator caspase-8 and then executioner caspase-3 to increase both cleavage form of PARP and nuclear DFF40 for DNA fragmentation.

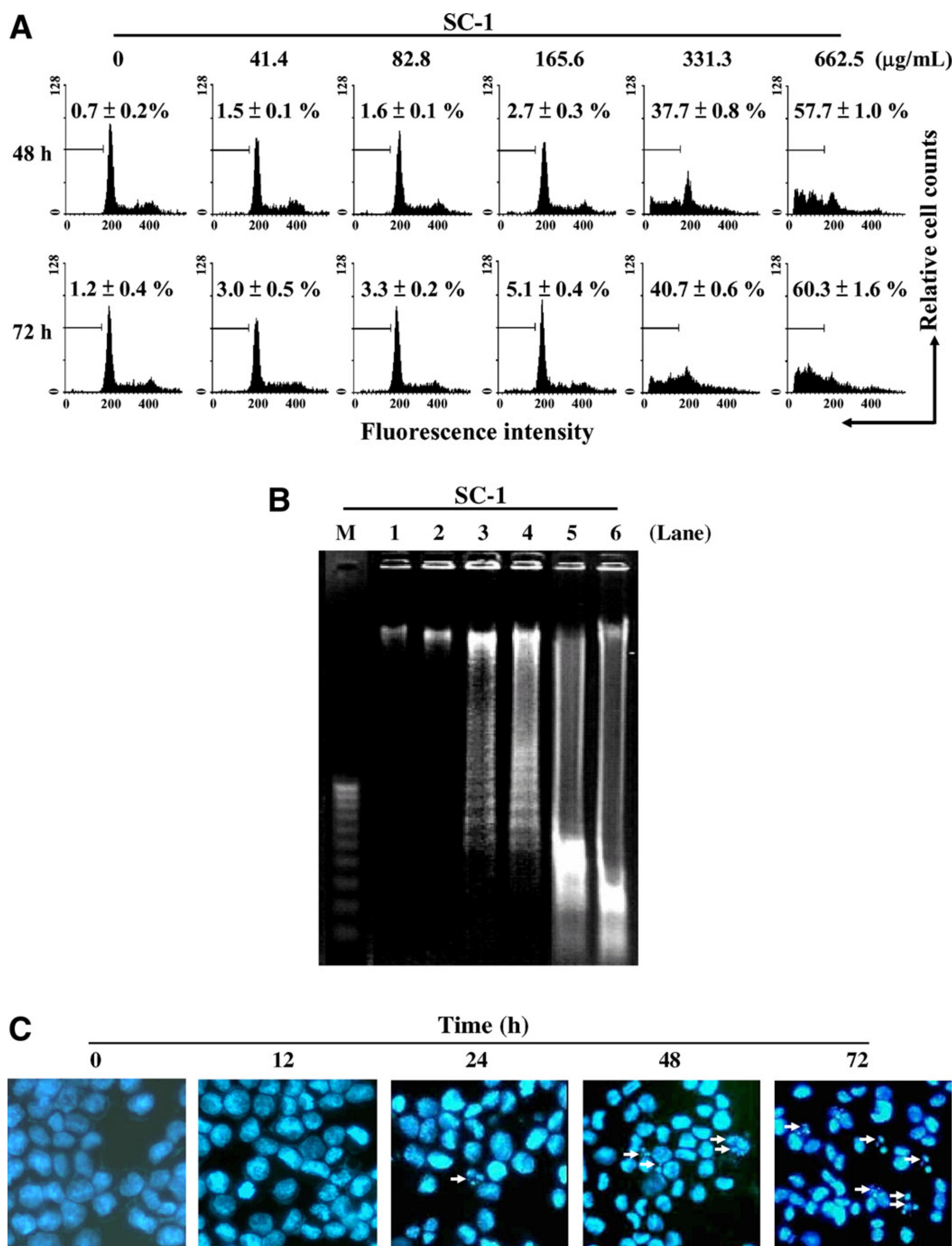

Fig. 1. Induction of apoptosis by SC-1 in Hep 3B cells. (A) Effect of SC-1 on cell cycle progression. Hep 3B cells (2×10^5 cells/well) were treated with the indicated concentrations of SC-1. At 48 and 72 h, cells were harvested and stained with PI for DNA content analysis. Apoptosis were measured by the accumulation of sub-G_1 DNA contents in the cells. The percentage in the figure indicates the proportion of apoptotic cells. (B) DNA fragmentation by SC-1. After treating with the indicated concentrations (0–662.5 μg/ml) of SC-1 for 72 h, Hep 3B cells (1×10^6 cells/well) were lyzed. DNA fragments were analyzed by 1% agarose gel electrophoresis. M, DNA molecular weight marker; lane 1, 0 μg/ml of SC-1; lane 2, 41.4 μg/ml of SC-1; lane 3, 82.8 μg/ml of SC-1; lane 4, 165.6 μg/ml of SC-1; lane 5, 331.3 μg/ml of SC-1; lane 6, 662.5 μg/ml of SC-1. (C) Change of nuclear morphology by SC-1. Hep 3B cells (1×10^5 cells/well) were treated with 165.6 μg/ml of SC-1 for 0–72 h. After staining with Hoechst 33258, morphology of the cells was analyzed by fluorescence microscopy. Arrowhead indicates apoptotic cells with condensed and segmented DNA. Dilutions were made by mixing SC-1 with DMEM. Control cells were treated with DMEM. Results are representative of three independent experiments.

3.4. Involvement of mitochondria in SC-1 induced apoptosis

Depolarization of mitochondrial membrane, regulated by the members of Bcl-2 family, has been reported could be an early event in apoptosis (Marchetti et al., 1996; Danial and Korsmeyer, 2004). To investigate if mitochondria anticipate in the process of SC-1 induced apoptosis, changes of $\Delta\psi_m$ were determined by staining cells with mitochondrial specific dye rhodamine 123, and the fluorescence

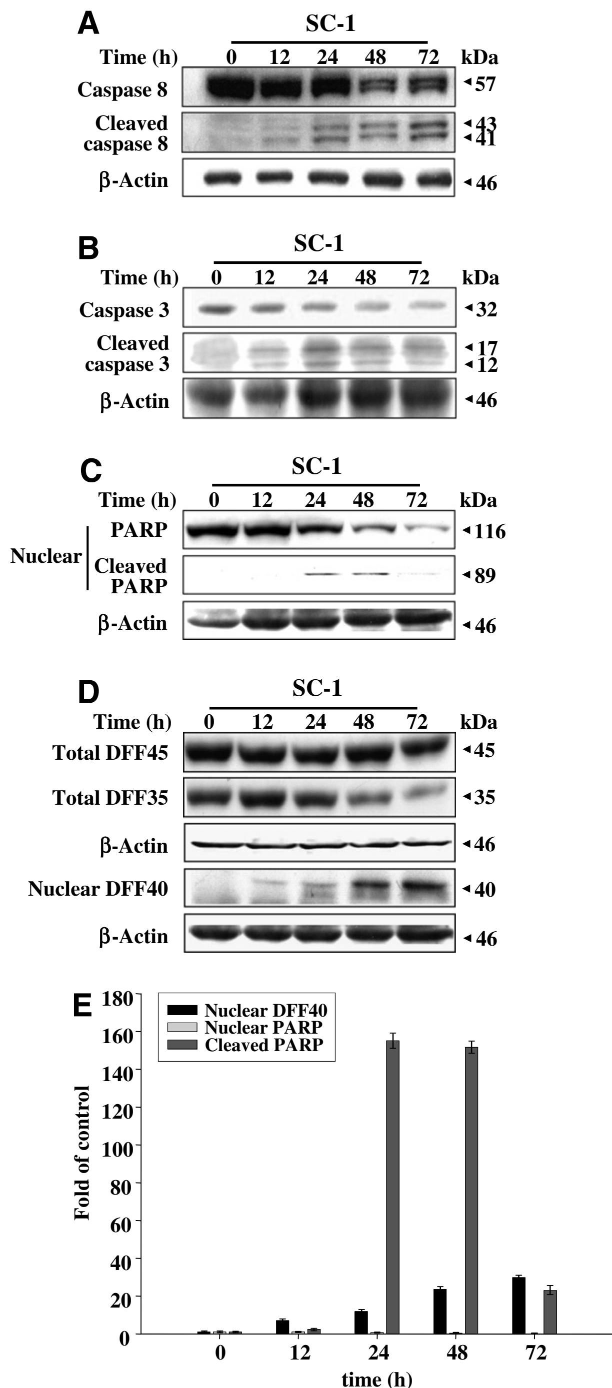

Fig. 2. Activation of caspases and their target proteins by SC-1. (A) caspase 8, (B) caspase 3, (C) PARP and (D) DFF expressions were determined in Hep 3B cells (5×10^5 cells/well) treated with 165.6 μg/ml of SC-1. At the indicated time periods (0–72 h), whole cell lysates or nuclear proteins were subjected for western blot analysis. Anti-caspase 8, anti-caspase 3, anti-PARP, anti-DFF45 (for total DFF) and anti-DFF40 (for nuclear DFF) antibodies were served as probes. β-actin was used as loading control. The intensity of individual nuclear PARP, cleaved PARP (C) and nuclear DFF40 (D) protein signal was quantified by densitometry. Each signal was normalizing to that of β-actin. The densities of nuclear PARP, cleaved PARP and DFF40 in the control condition were designated as 1, and the levels of the remaining samples were expressed as fold of the control (E). Results are representative of three independent experiments.

intensity was analyzed using confocal microscopy. As shown in Fig. 3, a time-related decrease in the intensity of rhodamine 123 staining was observed in the mitochondria of Hep 3B cells treated with SC-1 (165.6 μg/ml). The decrease in $\Delta\psi_m$ was observed as early as 12 h and reached the lowest level at 72 h (Fig. 3).

Expressions of Bcl-2 family proteins following SC-1 treatment were also evaluated. As shown in Fig. 4A and B, the increase in caspase 8-mediated cleavage of Bid into truncated Bid (tBid) was first observed at 12 h (1.4-fold) which is similar to the activation time of caspase 8 (Fig. 2A). The increase of mitochondrial tBid was peaked at 72 h of SC-1 (165.6 μg/ml) treatment (5.4-fold). Mitochondrial Bax was elevated at 48 h (2.0-fold) and kept increased at 72 h (3.0-fold) (Fig. 4A and B). Profoundly increase in mitochondrial Bak was observed as early as 12 h (37.7-fold) and peaked at 48–72 h (about 180-fold) in SC-1 treated Hep 3B cells (Fig. 4A and C). In contrast, SC-1 decreased anti-apoptotic Bcl-2 and Bcl-x_L in mitochondria at 24 h (0.6-fold) and 12 h (0.8-fold), respectively (Fig. 4A and B). Ku70 is also an important factor for apoptosis (Mancinelli et al., 2006). Decrease in Ku70 has been reported to enhance Bax-mediated apoptosis (Nothwehr and Martinou, 2003), while increase in Ku70 inhibits the process (Sawada et al., 2003). In Fig. 4D and E, SC-1 suppressed Ku70 in cytosol at 4–24 h. However, SC-1 did not change the expression of nuclear Ku70 (Fig. 4D). Reports also indicate that change of $\Delta\psi_m$ triggers the release of apoptogenic proteins such as cytochrome *c* and Smac (Hengartner, 2000; Roth and Reed, 2002). Complex of cytochrome *c*, apoptotic protease activating factor-1 (Apaf-1) and caspase-9 activates caspase-9 itself and thence caspase-3 (Hengartner, 2000). Smac also promotes caspase-9 activation by binding with XIAP to neutralize its anti-apoptotic activity (Shi, 2001). As expected, the release of cytochrome *c* (1.5, 2.0 and 4.0-fold at 12, 24 and 48 h, respectively) and Smac (1.5-fold at 48 and 1.9-fold at 72 h) from mitochondria to cytosol was observed (Fig. 4F and G). Decrease in XIAP (0.4-fold) was displayed at 12 h and reached the lowest level (0.1-fold) at 72 h in SC-1 (165.6 μg/ml) treated Hep 3B cells (Fig. 4F and G). After having shown that mitochondria were affected by SC-1, activation of caspase 9 was determined. As shown in Fig. 4H, expression of full length caspase 9 decreased and the activation form of cleaved caspase 9 increased in SC-1 (165.6 μg/ml) treated Hep 3B cells.

3.5. Changes of COX protein expression in SC-1 treated Hep 3B cells

Overexpression of COX-2 has been found in many types of cancer, and expression of HBV surface protein further increases the expression of COX-2 (Hung et al., 2004). As shown in Fig. 5A, COX-1 protein expressions in SC-1 treated Hep 3B cells were not affected. However, the expressions of COX-2 were significantly inhibited by the treatment of SC-1 (165.6 μg/ml) in a time-related manner

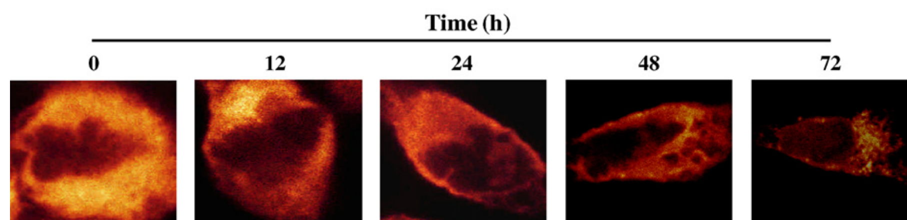

Fig. 3. Change in mitochondrial membrane potential ($\Delta\psi_m$). Hep 3B cells (1×10^5 cells/well) were treated with 165.6 μg/ml of SC-1 for 0–72 h. After staining with rhodamine 123, images of the cells were analyzed by confocal microscopy. Results are representative of three independent experiments.

(0.8, 0.5, 0.1-fold at 12, 24 and 48 h, respectively) (Fig. 5B and C).

4. Discussion

SC-1 effectively inhibits the growth and clonogenecity of human HCC (Hep 3B), mouse hepatoma cells (ML-1) and human colorectal carcinoma (HCT 116 and HT-29) cells (Table 1). Since colony formation is an *in vitro* assay used to examine anchorage-independent growth which strongly correlates with tumorgenicity and invasiveness of tumor cells (Moore et al., 1998), the data in Table 1 indicate SC-1 both affects anchorage-dependent and anchorage-independent growth of these tumor cells. Cytotoxicity of SC-1 on HBV-related human HCC Hep 3B cells is further investigated. The results in Fig. 1A–C reveal that SC-1 induces apoptosis in Hep 3B cells. Since the percentage of hypodiploid DNA pick represents the percentage of reduced DNA content of apoptotic nuclei which can be used to measure the percentage of cells with hypodiploid DNA content (apoptotic cells) (Nicoletti et al., 1991), the decrease in the accumulation of cells at sub-G_1 phase (Fig. 1A) suggests that the SC-1 treated Hep 3B cells is undergoing apoptotic process. Upon apoptotic induction, chromatin becomes condensed, apoptotic body forms, and activated endonucleases cleave DNA at the linker regions between nucleosomes to produce 180 bp oligonucleosome (DNA ladder) (Wyllie, 1980; Compton, 1992). Ladders of DNA and condensation of chromatin in SC-1 treated Hep 3B cells (Fig. 1B and C) provide further signs of apoptosis.

A cascade of proteolytic activity is involved in the process of apoptosis, much of which is performed by caspases (Earnshaw et al., 1999). Activation of caspase 8 can be triggered by death receptor-independent apoptotic stimuli including ionizing radiation, chemotherapeutic drugs and viruses (Green, 1998; Slee et al., 1999; Borner, 2003). Activated caspase 8 can directly activate its downstream effecter caspases, such as caspase 3 (Danial and Korsmeyer, 2004). PARP, a substrate of caspase 3, is reported to play a pivotal role in DNA repair mechanism (Sakahira et al., 1998; Soldani et al., 2001). DFF45 has also been reported to mediate genomic DNA fragmentation during apoptosis and can be degraded by activated caspase 3 to allow DFF40 entering the nucleus to execute DNA fragmentation (Chen et al., 2000). In the present study, both caspase 8 and 3 are activated at 12 h in SC-1 treated Hep 3B cells (Fig. 2A and B). The activated caspase 3 causes the release of DFF40 to enter the nucleus at 12 h (Fig. 2D and E), and also cleaves PARP at 24 h (Fig. 2C and E). The combination of the increase in nuclear cleaved PARP and DFF40 (Fig. 2C–E) may contribute to the fragmentation of DNA in SC-1 treated Hep 3B cells (Fig. 1B).

Activation of caspase-8 can also be stimulated by death receptor-dependent pathway. Fas has been reported to control programmed cell death in hepatocytes (Natoli et al., 1995) and play a essential role in the pathogenesis of liver diseases including hepatitis, cirrhosis and HCC (Galle et al., 1995). Upregulation of membrane Fas and Fas-L expression and induction of Fas-dependent apoptosis by chemotherapeutic drugs is revealed (Muller et al., 1997). In the present study, the expression of Fas and Fas-L was not affected by SC-1 in Hep 3B cells (data not shown). The percentages of cells accumulated at the sub-G_1 phase were not changed by an anti-Fas neutralizing ZB-4 antibody (data not shown). These results suggest the induction of apoptosis by SC-1 in Hep 3B cells is Fas-independent.

Accumulating evidences also suggest that caspase 8 acts as an upstream caspase to induce caspase 8-mediated cleavage of Bid into tBid, which triggers the activation of mitochondrial pathway and subsequently causes the release of cytochrome *c* to induce apoptotic signal (Wieder et al., 2001; Wajant, 2002). In the mitochondrial pathway, apoptogenic factors such as cytochrome *c* and Smac are released from the intermembrane space of mitochondria into the cytoplasm (Liu et al., 1996; Kroemer et al., 1997; Green, 1998; Du et al., 2000). Members of Bcl-2 family can either induce (Bid, Bax, Bad, Bik and Bak) or inhibit (Bcl-2, Bcl-w, Mcl-1 and Bcl-x_L) apoptosis through mitochondria controlling mechanism (Adams and Cory, 1998). Activation of Bid by caspase 8 links intrinsic and extrinsic apoptotic pathways through mitochondrial damages to activate the downstream caspases (Green and Reed, 1998; Li et al., 1998; Newmeyer and Ferguson-Miller, 2003). In addition, tBid triggers the oligomerization of pro-apoptotic Bak or Bax and results in the release of apoptogenic factors from mitochondria (Wei et al., 2001; Tsujimoto, 2003). However, Ku70 binds to pro-apoptotic protein Bax, prevents Bax translocate to the mitochondria and therefore inhibits apoptosis (Sawada et al., 2003). In the present study, the activated caspase 8 cleaves Bid into tBid at 12 h of SC-1

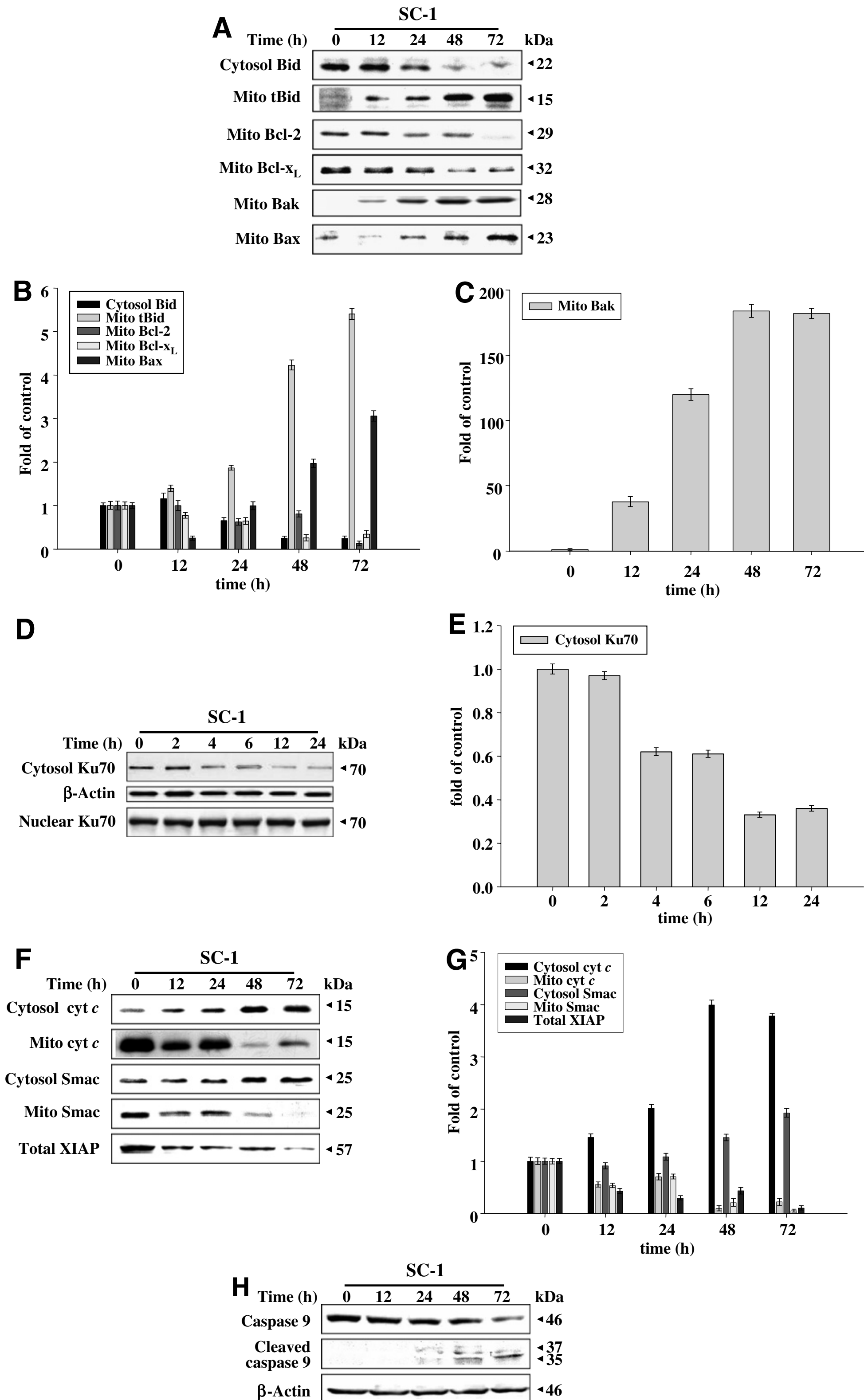

Fig. 4. Expressions of mitochondria-related proteins. (A) Distribution of Bid, tBid, Bcl-2, Bcl-x_L, Bak and Bax, (D) Expression of Ku70, (F) Distribution of cytochrome *c* (cyt *c*), Smac and XIAP, and (H) Expression of caspase 9 were determined by western blot analysis. Hep 3B cells (5×10^5 cells/well) were treated with 165.6 μg/ml of SC-1. At the indicated time periods (0–72 h), whole cell, mitochondrial (Mito), cytosolic and nuclear fractions were subjected for western blot analysis. Anti-Bid, anti-Bcl-2, anti-Bcl-x_L, anti-Bak, anti-Bax, anti-Ku70, anti-cytochrome *c*, anti-Smac, anti-XIAP and anti-caspase 9 antibodies were served as probes. β-actin was used as loading control. The intensity of each protein band in (A), (D) and (F) was quantified by densitometry. The densities of these proteins in the control condition were designated as 1, and the levels of the remaining samples were expressed as fold of the control (B), (C), (E) and (G). Results are representative of three independent experiments.

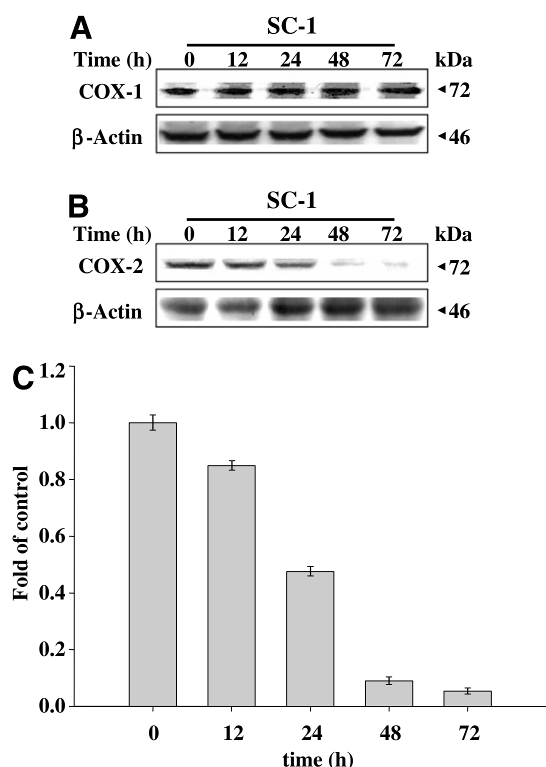

Fig. 5. Expressions of COX-1 and COX-2. (A) COX-1 and (B) COX-2 expressions were determined in Hep 3B cells (5×10^5 cells/well) treated with 165.6 μg/ml of SC-1. At the indicated time periods (0–72 h), whole cell lysates were subjected for western blot analysis. Anti-COX-1 and anti-COX-2, antibodies were served as probes. β-actin was used as loading control. The intensity of individual COX-2 (B) protein signal was quantified by densitometry. Each signal was normalizing to that of β-actin. The densities of COX-2 in the control condition were designated as 1, and the levels of the remaining samples were expressed as fold of the control (C).

treatment (Fig. 4A and B). The translocated tBid on the mitochondria (Fig. 4A and B) may form a complex with pro-apoptotic Bax or Bak (Fig. 4A–C) to disrupt $\Delta\psi_m$ and in turn trigger cytochrome *c* and Smac release from the mitochondria to the cytosol. Moreover, a time- and dose-related elevation of mitochondrial Bak (37.7-fold at 12 h) and Bax (2.0-fold at 48 h) indicates Bak is more important on the changes of $\Delta\psi_m$ compared with Bax (Fig. 4A–C). The significant decrease in cytosolic Ku70 (Fig. 4D and E) by SC-1 also suggests the increase in mitochondrial Bax (Fig. 4A) is via decrease in cytosolic Ku70. The loss of mitochondrial staining by SC-1 (Fig. 3) indicates the disruption of $\Delta\psi_m$, which may result from the changes in permeability of inner mitochondrial membrane (Kroemer et al., 1997). Colocalization of Bcl-2 family proteins at the surface of mitochondria has been reported to regulate the movement of cytochrome *c* (Hengartner, 2000). The release of cytochrome *c* and Smac is observed at 12 and 48 h (Fig. 4F and G). Since Smac can antagonize the anti-apoptotic function of XIAP, decrease in XIAP (0.4-fold at 12 h) may further promote the process of SC-1 induced apoptosis (Fig. 4F and G). Cytochrome *c* has been reported to bind and activate Apaf-1, which then aggregates caspase 9 to form apoptosome and thus activates caspase 9 and its downstream caspases such as caspase 3 (Nicholson, 2001; Roth and Reed, 2002). In the present study, the released cytochrome *c* may form apoptosomes to activate caspase 9 at 24 h (Fig. 4H). Activated caspase 9 further promotes the activation of caspase 3 (Fig. 2B) and its downstream regulators DFF40 and PARP (Fig. 2C–E). Since Bcl-x_L can bind to Bax and prevent the insertion of Bax into the outer membrane of mitochondria (Desagher and Martinou, 2000), the decrease of mitochondrial anti-apoptotic Bcl-2 at 24 h and Bcl-x_L at 12 h further supports the process of programmed cell death (Fig. 4A–C).

Regulation of apoptosis can also be made by inhibitors of COX proteins (Belka et al., 2004). Two major isoforms of COX were reported (Smith et al., 1996). COX-1 is expressed constitutively, while COX-2 can be induced at site of inflammation (Dubois et al., 1998) and contributes to carcinogenesis and resistance of apoptosis (Tsujii and DuBois, 1995). Increase in COX-2 is also related to increase metastasis of tumor cells (Jiang et al., 2001). In the present study, the protein levels of COX-2 in HBV-related Hep 3B cells are significantly inhibited by SC-1 in a time-related manner (Fig. 5B and C). Integration of HBV DNA into the genome of hepatocytes may cause transformation of the cells (Bruix and Llovet, 2003). X protein of HBV may induce COX-2 gene expression in HBV-infected liver cells and therefore increases risk of liver cancer (Cheng et al., 2004). Induction of apoptosis plus inhibition of COX-2 expression in Hep 3B cells offers SC-1 a good chance on chemoprevention and chemotherapy of HBV associated chronic liver disease and HBV-related HCC.

Interesting enough, all of the tested tumors are affected by the treatment of SC-1. Our previous experiment indicated that SC-1 (132.5 μg/ml) does not cause mutagenicity on *Salmenella typhimurium* tested strains (TA97, TA98, TA100, TA102 and TA1535) by Ames test, or clastogenicity in Chinese hamster ovary cells by *in vitro* chromosome aberration assay (unpublished data). In addition, no observed toxic effects are found in our parallel *in vivo* study, in which the soybean fermentation products were fed to BALB/c mice for 75 days and the tumor size was significantly reduced (unpublished data). Even though soybean products are thought in general to be relatively safe, the toxicity of SC-1 on normal cells awaits further assessment. The possible ingredients of soybean that account for cancer chemoprevention include isoflavones (genistein, genistin, daidzein and biochanin A), phytosterols, soy phytates, protease inhibitors and saponins (Chang et al., 2002). In primary human food, soybean is the predominant source of isoflavonoids, and which can only occur by dietary intake in mammals (Birt et al., 2001). Isoflavonoids, mostly present in foods as glycosidic conjugates, require enzymatic cleavage of the sugar moiety by mammalian or microbial glucosidases before absorption (Ohta et al.,

2000). Fermentation of soybean by microorganisms may alter the structure of the active compounds and therefore increase the availability of isoflavones in soy (Hutchins et al., 1995). SC-1 is a soybean product fermented with *B. subtilis* and *B. brevis*. So far, *Bacillus* species are still dominant bacteria in industrial fermentations, and some of them are on the Food and Drug Administration's GRAS (generally regarded as safe) list (Green et al., 1976). Possible products secreted by *Bacillus* species include enzymes, heterologous proteins, antibiotics, purine nucleotides, poly-γ-glutamic acid and D-ribose (Schallmey et al., 2004). Recently, a saturated branched-chain fatty acid, 13-methyltetradecanoic acid, other than isoflavonoids and other known compounds is isolated from soy fermentation products, and it exhibits significant antitumor effects by induction of apoptosis (Yang et al., 2000). Our previous experiment revealed that SC-1 contains total phenolic compounds (35.7 mg gallic acid equivalents/g dw), folic acid (8.4 ng/g dw), pantothenic acid (0.4 ng/g dw), vitamin B_6 (0.9 mg/g dw) and flavonoids (9.8 μg/g dw). In addition to the protective effects of flavonoids and phenolic compounds against carcinogenesis, the chemopreventive abilities of folic acid (Levin, 1999; Cao et al., 2005) and vitamin B6 (Komatsu et al., 2001; Komatsu et al., 2002) have recently been reported.

In conclusion, SC-1 serves as antitumor agents to inhibit the growth and clonogenesity of Hep 3B cells by induction of apoptosis. Molecular mechanism includes activation of caspase 8 and caspase 3, and increase of nuclear cleaved PARP and DFF40 expression. Increase of tBid, Bak and Bax on the mitochondria, decrease of mitochondrial anti-apoptotic Bcl-2 and Bcl-x_L proteins, disruption of $\Delta\psi_m$, release of mitochondrial apoptogenic proteins (cytochrome *c* and Smac), and the activation of caspase 9 in mitochondrial pathway also participate in the process of SC-1 induced programmed cell death. Decrease of Ku70 and COX-2 expression in Hep 3B cells further assists the process of apoptosis.

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