Endoplasmic reticulum stress mediates sulforaphane-induced apoptosis of HepG2 human hepatocellular carcinoma cells

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Received November 23, 2015; Accepted November 4, 2016

DOI: 10.3892/mmr.2016.6016

Abstract. Sulforaphane (SFN) is a naturally occurring chemopreventive agent, which effectively inhibits proliferation of HepG2 human hepatocellular carcinoma cells via mitochondria-mediated apoptosis. Endoplasmic reticulum stress is considered the most important cause of cell apoptosis; therefore, the present study aimed to determine whether the endoplasmic reticulum pathway was involved in SFN-induced apoptosis of HepG2 cells. An MTT assay was used to detect the inhibitory effects of SFN on HepG2 cells. Fluorescence microscopy was used to observe the morphological changes in apoptotic cells, and western blot analysis was conducted to detect the expression of binding immunoglobulin protein (Bip)/glucose-regulated protein 78 (GRP78), X-box binding protein-1 (XBP-1) and BH3 interacting domain death agonist (Bid). Furthermore, flow cytometry was used to determine the apoptotic rate of HepG2 cells, and the protein expression of C/EBP homologous protein (CHOP)/growth arrest- and DNA damage-inducible gene 153 (GADD153) and caspase-12 in HepG2 cells. The results indicated that SFN significantly inhibited the proliferation of HepG2 cells; the half maximal inhibitory concentration values were 32.03±0.96, 20.90±1.96 and 13.87±0.44 µmol/l, following treatment with SFN for 24, 48 and 72 h, respectively. Following 48 h of SFN treatment (10, 20 and 40 µmol/l), the apoptotic rates of HepG2 cells were 31.8, 61.3 and 77.1%, respectively. Furthermore, after 48 h of exposure to SFN, the cells presented typical morphological alterations of apoptosis, as detected under fluorescence microscopy. Treatment with SFN for 48 h also significantly upregulated the protein expression levels of Bip/GRP78, XBP-1, caspase-12, CHOP/GADD153 and Bid in HepG2 cells. In conclusion, endoplasmic reticulum stress may be considered the most important mechanism underlying SFN-induced apoptosis in HepG2 cells.

Introduction

Liver cancer is one of the most common malignancies worldwide. In China in 2012, liver cancer accounted for 12.9% of all novel cancer cases, 17.4% of all cases of cancer-associated mortality, and ranked second in cancer incidence and third in mortality rate (1). According to the latest data issued by the World Health Organization, liver cancer is the second most frequent cause of cancer-associated mortality worldwide (2). Several risk factors for liver cancer have been identified, including hepatitis B and C virus, aflatoxin, alcohol consumption, tobacco smoking, obesity and diabetes. Despite primary and secondary preventative measures, such as effective health education, hepatitis B vaccination and early detection in numerous areas of China, liver cancer mortality has only increased (3). The current treatment options for human hepatocellular carcinoma include surgery, radiotherapy, chemotherapy and liver transplantation, with limited evidence of a successful cure. The hepatosis of patients with hepatocellular carcinoma affects drug metabolism, which intensifies the side effects of chemotherapy and may induce multidrug resistance. Therefore, novel therapeutic options for human hepatocellular carcinoma have focused on natural products as an increasingly important source of potential anticancer agents that target liver cancer (4).

Sulforaphane (SFN) is a natural isothiocyanate that is present in cruciferous plants, with the highest content found in broccoli. SFN has received a great deal of attention due to its chemopreventive activity and potent anticancer effects (5). The chemopreventive activity of SFN has been investigated in various chemically induced cancer models (6). SFN modulates the metabolism of carcinogens via inhibition of cytochrome P450-dependent monooxygenases and/or induction of Phase II detoxification enzymes in chemically induced cancer. Previous studies have also demonstrated that SFN may inhibit proliferation of cancer cells in vitro by inducing apoptosis and/or cell cycle arrest (7-9). SFN suppresses growth in PA-1 human ovarian cancer (10), LNCaP and PC-3 human prostate cancer (11), T24 human urinary bladder cancer (12), pre-B acute lymphoblastic leukemia (ALL) (Nalm-6, REH and RS-4), and T-cell ALL cells (Jurkat, RPMI, DND41 and KOPTK1) (13). In PC-3 prostate cancer cells, SFN has been revealed to arrest cancer cells at the G2/M phase, which is associated with checkpoint kinase 2-mediated phosphorylation of cell division...
cytology 25C, and further induces caspase-9 and -8-mediated apoptosis (14). Furthermore, SFN reduces ovarian cancer cell migration and increases apoptotic cell death via increased B-cell lymphoma 2 (Bcl-2) antagonist killer/Bcl-2 ratio, and cleavage of procaspase-9 and poly (adenosine diphosphate-ribose) polymerase (15). Several studies have reported that SFN exerts a relatively strong effect on HepG2 human liver cancer cells, and evidently inhibits the proliferation of HepG2 cells (16-19).

A previous study demonstrated that SFN induces apoptosis of HepG2 cells via the mitochondrial pathway, through unknown molecular mechanisms (20). Endoplasmic reticulum (ER) stress is known to serve an important role in apoptosis mediated by several anticancer agents. However, whether SFN induces apoptosis of HepG2 cells via the ER pathway remains unclear. The present study aimed to explore the antiproliferative and apoptotic effects of SFN, and to determine the underlying mechanisms in HepG2 human liver cancer cells. SFN is transported by relevant proteins of the ER pathway; therefore, the role of the ER in SFN-induced apoptosis of HepG2 cells was explored.

Materials and methods

Reagents, drugs and assay kits. SFN (purity, 98.3%) was purchased from Alexis Biochemicals; Enzo Life Science (Farmingdale, NY, USA). The following additional materials were obtained: Adriamycin (ADR; 110826; Zhejiang Hisun Pharmaceutical Co., Ltd., Taizhou, China); RPMI-1640 culture medium and penicillin (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA); Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection kit, rabbit anti-human binding immunoglobulin protein (Bip)/glucose-regulated protein 78 (GRP78) antibody (cat. no. AF0171), precasted protein molecular weight marker (cat. no. P0062), cell lysis buffer, and alkaline phosphatase-labeled sheep anti-rabbit immunoglobulin protein (Bip) (FITC) Apoptosis Detection kit, rabbit anti-human binding protein-1 (XBP-1; cat. no. bs-1668R), BH3 interacting protein (CHOP) binding protein-1 (XBP-1; cat. no. bs-23014R), and FITC-conjugated goat anti-rabbit antibodies (cat. no. bs-0295G-FITC; BIORSS, Beijing, China).

Equipment. The following laboratory equipment was used throughout the experiments of the present study. Super-clean bench (JJT-900/1300; Suzhou SuJie Purifying Equipment Co., Ltd., Suzhou, China); microplate reader (Model 680; Bio-Rad Laboratories, Inc., Hercules, CA, USA); electrophoresis apparatus (DYY-24D, Beijing Liuyi Instrument Factory, Beijing, China); high-speed centrifuge at low temperature (Beckman Coulter, Inc., Brea, CA, USA); flow cytometer (COULTER® EPICS® XL™; Beckman Coulter, Inc.); CO₂ incubator (MC0175; SANYO Electric Co., Ltd., Moriguchi, Japan); and gel imaging system (GIES-2019, Tanon Science & Technology Co., Ltd., Shanghai, China).

Cell line and cell culture. The HepG2 human hepatocellular carcinoma cell line was obtained from the Research Center of Life Sciences and Environmental Sciences, Harbin University of Commerce (Harbin, China). HepG2 cells were maintained in culture flasks containing RPMI-1640 supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.). The cultures were incubated at 37°C in an atmosphere containing 5% CO₂ with saturated humidity. Cells were transferred to fresh medium once every 2 to 3 days.

MTT assay. Logarithmic phase HepG2 cells were seeded in a 96-well plate at a density of 5x10⁴ cells/ml (100 µl/well). Following a 24 h incubation at 37°C, 100 µl of the drugs were added to each well. The final concentrations of SFN used were 2.5, 5, 10, 20, 40 and 80 µmol/l; the final concentrations of ADR were 0.01, 0.1, 1, 10 and 100 µmol/l. Following treatment with SFN or ADR for 24, 48 and 72 h, drugs were discarded and the cells were incubated with 100 µl MTT (0.5 mg/ml) for 4 h at 37°C. After 4 h, the supernatant was aspirated, and 200 µl dimethyl sulfoxide was added. Finally, the absorbance was measured at 570 nm using a microplate reader, which was used to calculate the rate of inhibition and the half maximal inhibitory concentration (IC₅₀).

Fluorescence microscopy of apoptosis in HepG2 cells. After placing coverslips in a 6-well plate, 3x10⁴ HepG2 cells (1 ml) were seeded in each well and allowed to attach overnight. Cells were cultured with various concentrations of SFN (10, 20 and 40 µmol/l). ADR was added as a positive control at a final concentration of 0.5 µmol/l, and the control group was supplemented with equal volumes of RPMI-1640 culture medium. Following 48 h of treatment with SFN or ADR at 37°C, the cells were digested with pancreatin and washed once with phosphate-buffered saline (PBS). The cells were then fixed with a fixing solution composed of methyl alcohol and glacial acetic acid (3:1) for 10 min at 4°C. After further washing with PBS, 5 mg/l Hoechst 33258 fluorescent probe was added to the cells and incubated for 15 min. The cover slips were hand washed with PBS and placed on glass slides containing drops of glycerin. Finally, the cells were visualized and images were captured under an inverted fluorescence microscope.

Detection of apoptotic rate of HepG2 cells by flow cytometry (FCM). Logarithmic phase HepG2 cells were seeded in 6-well plates at a density of 3x10⁴ cells/ml (1 ml/well) and were allowed to attach overnight. SFN was added to the wells (1 ml per well) at a final concentration of 10, 20 or 40 µmol/l. ADR was added as a positive control at a final concentration of 0.5 µmol/l. An equal volume of medium was added to the wells of the control group. The plates were incubated at 37°C in an atmosphere containing 5% CO₂ for 48 h. The cells were digested with pancreatin, collected and washed with PBS (4°C), and adjusted to 1x10⁴ cells/ml. The cells were resuspended in binding buffer and then stained with Annexin V-FITC and propidium iodide (PI) solution in an ice bath in the dark, according to
the Annexin V-FITC Apoptosis Detection kit instructions. A nylon mesh filter (300 µm) was used to filter the cell samples to ensure cells were detected at single cell suspension. Following filtration, the cell samples of each group were analyzed by a flow cytometer at a wavelength of 488 nm and the results were analyzed by Expo 32 ADC Analysis software (Beckman Coulter, Inc.).

Western blot analysis of Bip/GRP78, XBP-1 and Bid protein expression. The HepG2 cells were plated in culture flasks and allowed to attach overnight. The cells were treated with various concentrations of SFN (10, 20 and 40 µmol/l) or ADR (0.5 µmol/l). The cells of the control group were treated with an equal volume of medium. After 48 h of treatment, cells were collected, lysed, and proteins were extracted. The Bradford method was used to quantify protein content. Equal amounts of protein (2 µg/ml; 20 µl loading volume) from the various groups were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were transferred onto nitrocellulose membranes at 200 mA for 30 min. The membranes were then incubated in blocking buffer [5% nonfat dry milk in Tris-buffered saline containing Tween-20 (TBST)] for 2 h at room temperature, and the blots were incubated with rabbit anti-human Bip/GRP78, XBP-1 and Bid antibodies (1:200) and β-actin antibody (1:5,000) overnight at 4˚C. The membranes were rinsed twice with TBST, undergoing 10 min of oscillation with each wash, and were rinsed once with TBS for 10 min. Subsequently, the membranes were incubated with alkaline phosphatase-labeled anti-mouse IgG antibody (1:2,000 dilution) for 2 h at room temperature. The membranes were rinsed three times as aforementioned and were stained with a diaminobenzidine chromogenic system. Finally, images were captured using the gel imaging system, and the protein content was quantified and analyzed using a GIS-2019 gel imaging system software, version 3.14 (Tanon Science & Technology Co., Ltd.).

FCM of caspase-12 and CHOP/GADD153 expression. Logarithmic phase HepG2 cells were seeded in 6-well plates at a density of 3x10^5 cells/ml (1 ml/well) and were allowed to attach overnight. Following treatment with various concentrations of SFN (10, 20 and 40 µmol/l) or ADR (0.5 µmol/l) for 48 h, the cells were collected by centrifugation for 10 min at 4˚C at a speed of 558 x g and washed twice with PBS. The cells were then fixed with paraformaldehyde (500 µl) for 30 min at room temperature, collected by centrifugation for 5 min at room temperature, at a speed of 558 x g and then washed with PBS for 45 min. The cells were permeabilized with 200 µl PBS containing 0.1% Triton X-100 for 10 min and blocked with 200 µl blocking solution (PBS containing 5% BSA) for 60 min. After rinsing once with PBS, the cells were incubated with rabbit anti-human CHOP/GADD153 or rabbit anti-human caspase-12 antibodies (1:400) for 2 h at room temperature. Subsequently, the cells were washed once with PBS and incubated with FITC-labeled goat anti-rabbit secondary antibody for 60 min in the dark at room temperature. The samples were finally resuspended in PBS and filtered via a 300 µm nylon mesh filter. The protein content was analyzed using flow cytometry and Expo 32 ADC Analysis software (Beckman Coulter Inc.).

Statistical analysis. Data are presented as the mean ± standard deviation of three independent experiments. Data were analyzed using SPSS Software for Windows Version 11.5 (SPSS, Inc., Chicago, IL, USA). Data from the various groups were compared using one-way analysis of variance and Fisher's least significant difference test. P<0.05 was considered to indicate a statistically significant difference.

Results

Antiproliferative effects of SFN. Following treatment with various concentrations of SFN for 24, 48 and 72 h, the proliferation of HepG2 cells was effectively inhibited in a dose- and time-dependent manner. The IC_{50} values of SFN treatment were 32.03±0.96, 20.90±1.96 and 13.87±0.44 µmol/l following treatment for 24, 48 and 72 h, respectively (Fig. 1). The IC_{50} values of ADR were 0.78±0.05, 0.43±0.09 and
0.32±0.40 µmol/l following treatment for 24, 48 and 72 h, respectively.

Effects of SFN on HepG2 cellular morphology. Following exposure to various concentrations of SFN or 0.5 µmol/l of ADR for 48 h, HepG2 cells displayed typical apoptotic morphology, including chromatin condensation and the formation of apoptotic bodies (Fig. 2). These results indicated that HepG2 cells were undergoing apoptosis.

Effects of SFN on the apoptotic rate of HepG2 cells. FCM demonstrated that treatment with 10, 20, 40 µmol/l SFN or 0.5 µmol/l of ADR significantly increased the apoptotic rate of HepG2 cells ( Annexin V † PI ) compared with that of the control group ( Table I and Fig. 3 ).

Effects of SFN on the protein expression levels of Bip/GRP78, XBP-1 and Bid. Following treatment with 20 or 40 µmol/l SFN or 0.5 µmol/l ADR for 48 h, the protein expression levels of Bip/GRP78, Bid and XBP-1 were significantly increased ( P<0.01; Fig. 4 ).

Effects of SFN on the protein expression of CHOP/GADD153 and caspase-12. The expression levels of CHOP/GADD153 and caspase-12 were significantly higher in HepG2 cells following exposure to increasing concentrations of SFN or 0.5 µmol/l of ADR for 48 h compared with control cells ( P<0.01 or P<0.05 ). Protein quantities relative to the control were calculated and plotted as histograms. The results are presented in Figs. 5 and 6.

Discussion

SFN exerts chemoprotective effects due to its antitumor activity, and exhibits no clinically adverse effects; therefore, it has been the focus of intensive research worldwide (21,22). Previous studies have demonstrated that SFN may induce apoptosis of several tumor cell lines through different pathways, and it has been shown to significantly reduce the mitochondrial membrane potential of human gastric cancer cells (23-25). Furthermore, SFN has been reported to decrease the ratio of Bcl-2/Bcl-2-associated X protein, reduce the expression of Bcl-2 in HepG2 cells, and activate caspase-3, thus triggering apoptosis of tumor cells (26). In addition, SFN may induce tumor cell apoptosis via the mitochondrial pathways. SFN was shown to induce a significant reduction in the expression of phosphorylated-extracellular signal-regulated kinase (ERK) in HepG2 cells, inhibit ERK/mitogen-activated protein kinase signaling, and promote apoptosis of HepG2 cells in a dose-dependent manner (27).

The present study aimed to investigate whether the ER pathway is involved in SFN-induced apoptosis of HepG2 cells. The Bip/GRP78 protein triggers ER stress; under normal physiological conditions, Bip/GRP78 combines with inositol-requiring enzyme 1 (IRE1), protein kinase RNA-like endoplasmic reticulum kinase (PERK) and activating transcription factor 6 (ATF6) to maintain its stability in the ER. When cells are stimulated by an external signal, Bip/GRP78 is released from IRE1, PERK and ATF6. By increasing the levels of Bip/GRP78 protein expression, ER stress is ameliorated...
Figure 3. SFN induces apoptosis of HepG2 cells in vitro. Annexin V-propidium iodide double staining and flow cytometric analysis were used to detect the apoptosis of HepG2 cells induced by SFN. The experiment was performed in triplicate. ADR, Adriamycin; SFN, sulforaphane; FITC, fluorescein isothiocyanate.

Figure 4. Effects of SFN on Bip/GRP78, XBP-1 and Bid expression in HepG2 cells. (A) Following treatment with 10, 20 or 40 µmol/l SFN for 48 h, the expression levels of Bip/GRP78, XBP-1 and Bid were analyzed by western blotting. Medium was used as a vehicle control and ADR (0.5 µmol/l) was used as a positive control. The relative density of (B) Bip/GRP78, (C) XBP-1 and (D) Bid protein was calculated and statistically analyzed. *P<0.05 vs. the control; **P<0.01 vs. the control. ADR, Adriamycin; SFN, sulforaphane; Bip, binding immunoglobulin protein; GRP78, glucose-regulated protein 78; XBP-1, X-box binding protein-1; Bid, BH3 interacting domain death agonist.
through a self-regulatory mechanism (28). ER stress in cells is predominantly resolved via the ATF6 and XBP-1-mediated pathways. At relatively low levels of stress, the steady state of ER may only be restored via the activation of ATF6 proteolysis; however, at markedly high level of stress, XBP-1 system serves a major role. Therefore, increased expression of XBP-1 protein is a marker of overwhelming ER stress in cells (29). Such stress cannot be relieved through self-regulatory mechanisms and the role of apoptosis becomes predominant. The ER is able to independently induce cellular apoptosis. The results of present study demonstrated that 20-40 µmol/l SFN treatment markedly upregulated the protein expression levels of Bip/GRP78 and XBP-1. These findings suggested that SFN treatment induced ER stress of HepG2 cells and the overexpression of XBP-1 suggested ER stress reached a peak level and apoptosis was subsequently triggered by the ER signaling pathway.

Out of the caspase family proteins, only caspase-12 is present in the ER, which is the key element for mediating the stress response to apoptosis. Caspase-12 triggers the transcription and expression of CHOP/GADD153 (30). CHOP/GADD153 is minimally expressed in normal cells; however, under conditions of ER stress, its expression is increased, resulting in apoptosis (31). Bid is predominantly expressed in the ER, but also in the nucleus to a limited extent. Upon apoptotic signaling, Bid translocates to the mitochondria and increases mitochondrial membrane permeability, thus leading to the release of cytochrome c, activation of caspase-9, and induction of apoptosis (32). The results of present studies revealed that 20-40 µmol/l SFN treatment markedly upregulated the protein expression levels of caspase-12, CHOP/GADD153 and Bid. Following SFN treatment for 48 h, the apoptosis rate of HepG2 cells significantly increased. These findings suggested that SFN may induce ER stress-mediated apoptosis in HepG2 cells. A previous study demonstrated that SFN decreases the expression of Bcl-2 in HepG2 cells, but increases Bax levels, resulting in the release of cytochrome c and the enhanced
activity of caspase-3, resulting in the induction of apoptosis via the mitochondrial pathway (20). CHOP/GADD153 is a classic marker of ER stress and primarily induces apoptosis by inhibiting the expression of Bcl-2, which is downregulated in SFN-treated HepG2 cells. The overexpression of Bid induced by SFN may promote mitochondrial-mediated apoptosis and may be the primary mechanism underlying the induction of mitochondrial-mediated apoptosis by SFN.

In conclusion, in addition to directly inducing apoptosis of HepG2 cells via mitochondrial pathways, the present study demonstrated that SFN also triggers ER stress in HepG2 cells. By modulating ER-related protein expression, SFN activates the expression of Bid, which further activates mitochondrial apoptosis and induces cellular apoptosis. These results indicated that the mechanism underlying SFN-induced apoptosis is mediated by the interaction between the ER and mitochondrial pathways, and Bid serves an important role. Therefore, the ER pathway may be involved in SFN-induced HepG2 cell apoptosis. The induction of cell apoptosis is an important cancer therapeutic strategy. The present study demonstrated SFN was able to induce cell apoptosis via the ER stress-mediated pathway. SFN is considered a promising drug in the treatment of different types of cancer due to its chemopreventative and therapeutic effects in various cancer cells. The elucidation of the underlying molecular mechanisms of SFN provides novel evidence for the research and application of SFN-related anticancer therapeutic agents.

Acknowledgements

The present study was supported by the National Natural Science Funds of China (grant no. 81102858); the Postdoctoral Science Foundation (grant no. 2013M321061); the Key Project of Chinese Ministry of Education (grant no. 210059); the Heilongjiang Postdoctoral Fund (grant no. LBH-Z11102); the University Nursing Program for Young Scholars
with Creative Talents in Heilongjiang Province (grant no. UNPYSCT-2015070).

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