Involvement of apoptosis and autophagy in reducing mouse hepatoma ML-1 cell growth in inbred BALB/c mice by bacterial fermented soybean products

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Abstract

Followed by the results of our previous in vitro report (Food Chem. Toxicol., 2007), the efficacy of the soybean fermentation products containing live bacteria (SCB) was demonstrated using a syngeneic animal model. Murine HBV-related hepatoma ML-1 cells, derived from inbred animals and tumorigenic in BALB/c mice, were implanted subcutaneously to the flank of BALB/c mice on day 0. Three days after implantation, SCB (1.0 or 1.3 ml/mouse/day) or vehicle (water) was orally administrated daily until day 60. The results indicate that SCB significantly reduced (P < 0.05) the volumes and weights of tumors during the experimental periods. Examination using TUNEL staining on section of tumors revealed apoptotic phenomenon of nuclear DNA double-strand breaks in the groups of mice received SCB. Immunohistochemistry further revealed an autophagic LC3-II punctate pattern. Of note, SCB induced autophagy in the absence or presence of apoptosis, whereas, apoptosis was observed only in combination with autophagy. In vitro study using autophagy inhibitor indicated that the induction of autophagy promoted apoptosis. These data imply that the suppression in tumor volumes and tumor weights by oral administration of SCB was due to the induction of apoptotic and autophagic cell death, which suggests therapeutic potential of SCB on HBV-related HCC.

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1. Introduction

Human hepatocellular carcinoma (HCC) is the fifth most common cancer in the world, and the third among all cancers for mortality (Fattovich et al., 2004). Chronic infection of hepatitis B virus (HBV) increases the risk of HCC to a 100-fold compared with non-infected individuals (Beasley et al., 1981). Systematic treatments with standard chemotherapeutic agents provide only marginal benefit, and the recurrence rate remains high (Zhu, 2006).

Apoptosis (type I) and autophagy (type II) are two different types of programmed cell death (Shimizu et al., 2004). Apoptosis is an organized and energy-dependent process, which allows the organism to maintain tissue homeostasis (Saraste, 1999). Insufficient of apoptosis contributes to the pathogenesis of cancer (Thompson, 1995). Autophagy is also a normal physiological process which promotes cell adaptation and survival, but under some conditions it leads to cell death (Lockshin and Zakeri, 2004; Maiuri et al., 2007). The process of autophagy involves degradation and recycling of cell organelles and proteins in autolysosomes (a fusion compartment of autophagosomes and lysosomes) (Kondo et al., 2005). In response to therapy, cancer cells may undergo both apoptosis and autophagy (Kondo et al., 2005; Maiuri et al., 2007).

Dietary intake of soybean and soybean-based products has been reported to reduce risks of several cancers (Chang et al., 2002). Predominant source of flavonoids and isoflavonoids may be responsible for the antitumor mechanisms (Birt et al., 2001). Recent report indicates that administration of phytochemical compounds including flavonoids conjunction with chemotherapy enhances therapeutic efficacy (Alisi and Balsano, 2007). In addition, fermentation of soybeans by microorganisms may elevate the availability of isoflavonoids by changing their structures (Hutchins et al., 1995). Our previous published report has revealed that SC-1, the filtered (0.22 mm) aqueous phase of soybean fermentation products by bacteria Bacillus subtilis and Bacillus brevis, significantly inhibited...
the growth and clonogenicity of HBV-related HCC Hep 3B cells and mouse hepatoma ML-1 cells (Su et al., 2007). Cytotoxicity of SC-1 on cultured Hep 3B cells was due to the induction of caspase-8 and mitochondria-related apoptosis (Su et al., 2007). In the present study we further demonstrate that SCB, the soybean fermentation products containing live bacteria B. subtilis and B. brevis, significantly suppressed the growth of ML-1 cells in inbred BALB/c mice due to the induction of both apoptosis and autophagy in vivo using immunohistochemistry on representative sections of tumors.

2. Materials and methods

2.1. Preparation of soybean fermentation products

One kilogram of dried soybeans (Dongshi Shiang, Chiayi, Taiwan) were ground, boiled, and soaked in water for 10 days. After removing the big chunk of soybeans, the supernatant was fermented with B. subtilis (10^9 cells/ml) and B. brevis (10^6 cells/ml) for 1 month at 37 °C (Su et al., 2007). The soybean fermentation products containing live bacteria were used for animal study. For in vitro study, SC-1 was obtained by centrifuging the fermentation product at 15,000g for 30 min, and filtered through 0.22 μm filter (Corning, Corning, NY) to avoid the bacteria contamination (Su et al., 2007).

2.2. Cell culture

Human HCC Hep 3B cells and murine ML-1 cells obtained respectively from American Type Culture Collection (ATCC, Rockville, MD) and Dr. Huan-Yao Lei (Department of Microbiology and Immunology, Medical College, National Cheng Kung University, Tainan, Taiwan) were cultured in complete Dulbecco’s modified Eagle medium (DMEM; Gibco BRL, Grand Island, NY) containing 10% fetal bovine serum (Gibco BRL), 2 mM glutamine (Sigma, St. Louis, MO), 100 U/ml penicillin, 100 μl/ml penicillin (Sigma), and 100 μg/ml streptomycin (Sigma) at 37 °C in a 5% CO2 humidified atmosphere.

2.3. Animal study

As previously described (Lee et al., 2005), BALB/c mice at 6–7 weeks of age with body weight between 18 and 23 g were obtained from the Animal Center of the National Cheng Kung University (NCKU, Tainan, Taiwan). They were bred and housed at the Animal Center in a temperature-controlled and air-conditioned environment with a 10/14 h light/dark cycle. Food and water were provided ad libitum. ML-1 cells were implanted subcutaneously (s.c.; 2.5 × 10^6 cells/mouse) to the flank of BALB/c mice at day 0. The mice were randomly divided into three groups at day 4. Treatment groups of mice received oral administration of SCB (1.0 or 1.3 ml/mouse/day) for 56 consecutive days, and another group received vehicle (water) on the same schedule. Mice were monitored every other day for gross anatomical changes. Tumor growth was measured with a caliper every other day. Tumor volume was calculated by using the formula L × W^2/2, where L (length) and W (width) are in millimeters and L is greater than W (Chang et al., 1994). All animal experiments were approved by the Animal Research Committee of NCKU and were performed under the guidelines of the National Research Council, Taiwan (IACUC940047).

2.4. Immunohistochemistry

Tumors obtained from SCB- or vehicle-treated mice were frozen on liquid nitrogen and stored at -80 °C until use. Apoptosis was detected using terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling (TUNEL) method (ApoAlert DNA Fragmentation assay kit, Clontech, Palo Alto, CA). Apoptag was examined using anti-cleaved LC3 antibody (ABGENT, San Diego, CA; 1:300). Tumor sections were subjected to apoptosis and/or autophagy analysis. Briefly, tumor sections (5 μm) cut by Cryotome0620 (Thermo Shandon, Waltham, MA) were incubated with 3.7% formaldehyde (Sigma) for 1 min at room temperature and then with cold ethanol [Merck, Darmstadt, Germany]/acetic acid (Wako, Osaka, Japan; 2:1, v/v) for 5 min at -20 °C. Subsequently, the sections were quenched with 3% hydrogen peroxide (Wako) for 5 min, and subjected to TUNEL assay according to the manufacturer’s protocol and the incorporated fluorescein-dUTP at the free 3'-hydroxyl ends of fragmented DNA was detected. After washing, the sections were incubated with blocking buffer (SuperBlock Blocking Buffer, Thermo Scientific, Rockford, IL) for 30 min at room temperature. Immunostaining was carried out by incubating tumor sections with rabbit polyclonal anti-cleaved LC3 antibody (ABGENT, San Diego, CA; 1:300) for overnight at 4 °C and then with goat anti-rabbit Alexa Fluor568-conjugated secondary antibody (Molecular Probes, Inc., Eugene, OR; 1:450) in blocking solution for 2 h at room temperature. After washing, fluorescein-dUTP and/or anti-cleaved LC3 antibody stained sections were incubated with Hoechst 33258 (Sigma–Aldrich, St. Louis, MO; 0.05 μg/ml in PBS) for 10 min at room temperature. The signals were detected with a fluorescence microscope (OLYMPUS BX51).

2.5. Flow cytometric analysis of apoptotic cells

As previously described (Lee et al., 2005), cells (2 × 10^6) grown in 6-well plates were pretreated with or without 10 μM of autophagy inhibitor 3-methyladenine (3-MA; Sigma, St. Louis, MO) for 2 h prior to the addition of SC-1 (265 μg/ml). The cells were harvested, centrifuged at 800g for 10 min at 4 °C, and resuspended in PBS containing 40 μg/ml of propidium iodide and 100 μg/ml RNase A for 30 min at 37 °C in the dark. Measurement of apoptotic cells was performed using a FacScan flow cytometer (Becton Dickinson, Mountain View, CA, USA).

2.6. Statistical analysis

The results were expressed as means ± standard errors of the means (SEM). Differences in tumor volumes were analyzed by the Student’s t test (Minitab software, version 10.2). A difference was considered if P < 0.05. Weights of body, tumor, and liver were analyzed by One-way ANOVA. Differences among groups were analyzed by Duncan’s multiple range test (SPSS software, version 14.0). A difference was considered if P < 0.05.

3. Results

Our previous published report has demonstrated that SC-1 suppressed the growth and clonogenicity of hepatoma ML-1 cells in vitro (Su et al., 2007). The present study was carried out to determine the efficacy of SCB on the growth of ML-1 cells in vivo. Both SC-1 and SCB are fermented soybean products by bacteria B. subtilis and B. brevis. The main difference between these two is that SCB contains live bacteria but SC-1 does not. For the in vivo study, murine ML-1 cells were implanted s.c. to the flank of inbred BALB/c mice followed by oral administration of SCB (1.0 or 1.3 ml/mouse/day) or vehicle (water) for 56 consecutive days. The growth of ML-1 cells was monitored every other day until day 60. As shown in Fig. 1A, at day 30, the growth of ML-1 cells was apparent in the control mice received vehicle. In contrast, the growth of ML-1 cells in the mice received SCB (1.3 ml/mouse/day) was not visible. At day 60, the size of the tumor in the control mice became much larger compared with that at day 30, whereas that in the group of mice received SCB (1.3 ml/mouse/day) was not dramatically changed. During the experiment, growth of tumor was measured with a caliper every other day. Differences in tumor volumes were analyzed. As shown in Fig. 1B, administration of SCB (1.0 or 1.3 ml/mouse/day) significantly inhibited (P < 0.05) the size of tumors throughout the experimental period. Of note, all mice survived until the end of the experiment. No apparent illness was found in the mice received SCB. Body weights and liver weights were not significantly altered (P > 0.05) by SCB (Table 1).

Our published report also demonstrates that SC-1 inhibited the growth of cultured HCC Hep 3B cells via activation of apoptotic signaling cascades (Su et al., 2007). To confirm the induction of apoptosis in vivo, sections of tumors were subjected to TUNEL assay before fluorescence microscopy to examine the phenomenon of apoptosis, nuclear DNA double-strand breaks. As shown in Fig. 2A, viewed using fluorescence microscope at 100×, treatment of SCB increased positive TUNEL staining compared with the vehicle control. Fig. 2B, viewed at 400×, further reveals that the sections of tumors obtained from the mice received vehicle did not show apparent green apoptotic fluorescence at the nuclei, indicating that apoptotic events did not occur in the control. In contrast, the sections of tumors obtained from the mice received SCB (1.3 ml/mouse/day) exhibited intensive green fluorescence at the nuclei, representing nuclear DNA double-strand breaks, a hallmark of apoptosis (Nagata, 2000). To investigate other possible reasons for the reduction in tumor volumes (Fig. 1A and B) and tumor weights (Table 1), phenomena of autophagy were determined in the tumors. As shown in Fig. 3A, morphometric analysis of cleaved LC3 distribution viewed at 100× exhibits that SCB elevated cleaved LC3 staining compared with the control. Using fluorescence microscope viewed at 400× further discovered that no cytoplasmic...
cleaved LC3 was found around the nuclei in the control (Fig. 3B). In contrast, in the SCB-treated group, the expression of cleaved LC3 was increased and displayed a punctate staining, representing the location of autophagic marker LC3-II on autophagosomes (Kondo and Kondo, 2006). These results suggest that SCB induced both apoptosis and autophagy in ML-1 cells in vivo. Furthermore, the merge images of Hoechst 33258, TUNEL, and cleaved LC3 staining indicates that SCB induced autophagy alone without induction of apoptosis, characterized by blue nuclei surrounded with red LC3 puncta (Fig. 4A and B). Interesting enough, SCB did not induce apoptosis alone without induction of autophagy since almost every nucleus with green staining was surrounded with red cleaved LC3 puncta.

To confirm the importance of autophagy in the proceeding of apoptosis, autophagy inhibitor 3-MA was added to the cell culture and the relative portion of apoptotic cells was analyzed by flow cytometry. The results indicate that 3-MA suppressed SC-1-induced apoptosis in both murine hepatoma ML-1 and human HCC Hep 3B cells in a time-related manner. As shown in Fig. 5A, administration of 3-MA inhibited SC-1-induced apoptosis of ML-1 cells approximately 45% at 48 h and 52% at 72 h. In Fig. 5B, it inhibited SC-1-induced apoptosis of Hep 3B cells approximately 42% at 48 h and 60% at 72 h. Therefore, it would not be surprising that the induction of apoptosis was observed only in combination with autophagy in tumors obtained from the mice received SCB for a relatively long period of time (Fig. 4A and B).

### 4. Discussion

Results of the present study demonstrate that oral administration of SCB was capable of reducing the growth of murine hepatoma ML-1 cells in inbred BALB/c mice. SCB was well tolerated.
and did not alter mean body weights or mean liver weights. Decrease in the growth of ML-1 cells may be due to the induction of apoptosis and autophagy characterized by the breaks of nuclear DNA and formation of LC3 puncta, respectively. In vitro study further demonstrated that autophagy promoted SC-1-induced apoptosis since the inhibition of autophagy decreased the proportion of apoptotic murine hepatoma ML-1 and human HCC Hep 3B cells in response to SC-1. Severe combined immunodeficiency (SCID) mouse is characterized by the complete inability of the adaptive immunity due to the disruption in the differentiation of both B and T cells (Perryman, 2004). Nude mouse is a strain of athymic mouse characterized by greatly reduced number of T cells. Both mice do not reject tumor cells and therefore have been used for cancer research (Sharkey and Fogh, 1984). However, these mice are immunodeficient and require a pathogen-free environment. Since SCB contains live bacteria, SCID or nude mice may not survive if given SCB. BALB/c mice have normal immune function and were therefore chosen for SCB-related animal studies. ML-1 cell line was prepared by transfected primary BALB/c murine hepatocytes with plasmid DNA and HBV DNA (Chen et al., 1992). They were immortalized, derived from inbred animals, and tumorigenic in BALB/c mice (Chen et al., 1992). Unlike SCID or nude mice, the growth of ML-1 cells in inbred BALB/c mice, a syngeneic animal model, represents a balanced result between tumorigenic activities and host immune responses. Besides, ML-1 cells expressed HBV genes, secreted HBV antigens

**Fig. 2.** SCB induced apoptosis of ML-1 cells implanted to BALB/c mice. (A) Representative section of tumors viewed at 100×. (B) Representative section of tumors viewed at 400×. Sections of tumors were stained with Hoechst 33258 (blue) and fluorescein-dUTP (TUNEL assay; green) to visualize cell nuclei and apoptotic nuclei, respectively. Results are representative of three independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
and HBV-like particles into the culture medium (Chen et al., 1993), which may be a model to further study host responses both in vivo and in vitro against HVB gene-expressing hepatocytes in the presence and absence of SCB.

It has also been suggested that fermentation would increase bioavailability of isoflavonoides (Hutchins et al., 1995). Bacillus species are dominant bacteria in industrial fermentation, and some of them are on the Food and Drug Administration’s GRAS (generally regarded as safe) list (Green et al., 1976). In this study, neither apparent illness nor changes in mean body weights or mean liver weights was observed in mice received SCB, indicating SCB was relatively safe. Our unpublished results also indicate that no acute oral toxicity in ICR mice or in Sprague–Dawley rats was found when these animals were fed SCB up to 40 ml/kg/day for consecutive 14 days. Negative results were also revealed in micronucleus assay, in which the percentage of reticulocytes to total erythrocytes was not significantly changed when BALB/c mice were given with or without SCB up to 40 ml/kg/day for consecutive 5 days (unpublished data). The ingredients of SCB include phenolic compounds, folic acid, pantothenic acid, vitamin B6, and fermented products of \textit{B. subtilis} and \textit{B. brevis} (Su et al., 2007).

Our previously results have demonstrated that SC-1 induced apoptosis of HCC Hep 3B cells characterized by accumulation of the cells at sub-G\textsubscript{1} phase, fragmentation of DNA, and change of nuclear morphology (Su et al., 2007). The induction of apoptosis was via activation of caspase-8 which cleaved Bid into tBid to disrupt

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**Fig. 3.** SCB induced autophagy of ML-1 cells implanted to BALB/c mice. (A) Representative section of tumors viewed at 100×. (B) Representative section of tumors viewed at 400×. Sections of tumors were stained with Hoechst 33258 (blue) and anti-cleaved LC3 antibody (red) to visualize cell nuclei and autophagic punctate pattern of LC3-II, respectively. Results are representative of three independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
mitochondrial membrane potential, leading to the release of cytochrome c and anti-second mitochondria-derived activator of caspase/direct IAP binding protein with low PI (Smac/DIABLO) to further activate caspase-9 and -3. Interestingly, SC-1 also suppressed the expression of cyclooxygenase-2 (Su et al., 2007), indicating that SC-1 may also associate with inflammation (Dubois et al., 1998), carcinogenesis (Tsujii and DuBois, 1995), and metastasis (Jiang et al., 2001).

Autophagy, controlled by a group of evolutionarily conserved genes, has been found in all eukaryotic cells (Kondo et al., 2005). Anticancer therapies such as chemicals, irradiation, and hyperthermia induce autophagy and result in death (autophagic cell death) of breast, colon, prostate, and brain cancers (Kondo et al., 2005). However, autophagy might remove the proteins or organelles that are damaged by cancer therapy, and become protective to the treatment (protective autophagy). Although tumor cells can undergo both apoptosis and autophagy, the effect of autophagy in cancer cell death or survival is still unclear and sometimes contradictory (Apel et al., 2009; Eisenberg-Lerner et al., 2009; Loos and Engelbracht, 2009). Apoptosis and autophagy are not always separate and

Fig. 4. Induction of both apoptosis and autophagy in tumors. (A) Tumors obtained from the mice received oral administration of water (control group). (B) Tumors obtained from the mice received oral administration of SCB (1.3 ml/mouse/day). Sections of tumors were stained with Hoechst 33258 (blue), fluorescein-dUTP (TUNEL assay; green), and anti-cleaved LC3 antibody (red) to visualize cell nuclei, apoptotic nuclei, and autophagic punctate pattern of LC3-II, respectively. Results are representative of three independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
may be triggered by similar stimuli (Maiuri et al., 2007). There can be crosstalk between these two pathways. The molecular mechanisms involved in the contribution of autophagy in SC-1-induced apoptosis await further investigation. Unlike severe combined immunodeficiency (SCID) or nude mice, induction of apoptosis and autophagy leading to the reduction in the growth of ML-1 cells in BALB/c mice represents a balanced result between tumorigenic activities and host immune responses.

Taken together, oral administration of SCB suppresses the growth of ML-1 cells via induction of apoptosis and autophagy without significant changing the mean body and liver weights using a syngeneic animal model, indicating the safety and efficacy of SCB in vivo and suggesting chemotherapeutic potential of SCB on HBV-related HCC.

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Conflict of interest

The authors declare that there are no conflicts of interest.