Induction of macroautophagy in human colon cancer cells by soybean B-group triterpenoid saponins

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The impact of triterpenoid saponins isolated from soybeans on suppression of colon cancer cell proliferation was evaluated. Experiments were conducted to determine the effects of a purified soybean B-group saponin extract on cell proliferation, cell-cycle distribution and programmed cell death in cultures of human HCT-15 colon adenocarcinoma cells. Treatment of cells with the soyasaponins at concentrations of 25–50 p.p.m. significantly reduced viable cell numbers after 24 and 48 h of exposure. Treatment of cells with 25 and 100 p.p.m. of saponins also resulted in a transient accumulation of cells in the S-phase of the cell cycle that was associated with a significant reduction of cyclin-dependant kinase-2 (CDK-2) activity. More striking was that, when examined by transmission electron microscopy, soyasaponin-treated cells exhibited an ∼4.5-fold increase in cell morphologies characteristic of Type II non-apoptotic programmed cell death (PCD) including numerous autophagic vacuoles, changes that collectively suggest autophagic cell death. In addition, the protein levels of microtubule-associated protein light chain 3 (LC-3), a specific marker of macroautophagy, increased substantially following soyasaponin treatment. Taken together these results thus indicate that soybean saponins, at physiologically relevant doses, can suppress HCT-15 colon cancer cell proliferation through S-phase cell-cycle delay, and can induce macroautophagy, the hallmark of Type II PCD. These findings suggest that B-group soyasaponins may be another colon-cancer suppressive component of soy that warrants further examination as a potential chemopreventive phytochemical.

Introduction

Colon cancer is a leading contributor to cancer prevalence and mortality in the Western world, accounting for 15% of all cancers diagnosed annually in the US (1). Identification and application of effective prevention and treatment strategies for this disease represent crucial public health challenges to industrialized countries. Diet has been recognized as an important modifiable risk factor for colon cancer for decades (2).

Specifically, a consistent inverse association between diets rich in plant foods and colon cancer incidence has been identified through epidemiologic studies (3–5). Dietary patterns that emphasize legumes such as soybeans as protein alternatives to meat also may reduce cancer risk (6). For example, consumption of at least two servings of legumes per week has been associated with a reduced colon cancer incidence through prospective and case-control studies (7,8).

With regard to soy, limited epidemiologic evidence suggests an inverse association between soy food consumption and colon-cancer incidence (9). Of interest is a prospective double-blind intervention trial that indicated daily dietary inclusion of 39 g of soy protein isolates for 1 year reduced the recurrence of adenomatous polyps (10). Soybeans contain various non-nutritive compounds that potentially contribute to its anticancer effects, including phytoestrogens, phytosterols, protease inhibitors, inositol hexaphosphate and saponins. Although soy isoflavones have received considerable attention as putative anticancer agents, saponins are another group of soy constituents that may also have bioactivity in inhibiting cancer.

In animal models, dietary administration of foods that contain soyasaponins decreased chemically induced colon carcinogenesis. Specifically, ingestion of soy flour and soy flakes, products known to include soyasaponins, significantly reduced the incidence and the multiplicity of aberrant crypt foci in F344 rats, while soy protein concentrate, an ethanol-washed food component devoid of saponins, did not (11). Soy protein isolate, also known to contain soyasaponins, significantly reduced colon tumor incidence in Sprague-Dawley rats compared with casein-fed controls (12). In particular, post-initiation administration of a 3% crude soybean saponin diet to CF1 mice significantly reduced the incidence and multiplicity of aberrant crypt foci (13).

Saponins are amphiphilic compounds composed of watersoluble sugar residues attached to a lipid soluble aglycone (designated the sapogenol). The biological activity of each saponin depends upon its polarity, acidity and hydrophobicity, characteristics determined by chemical structure (14). Under in vitro conditions, various saponins have demonstrated antimutagenic, anticarcinogenic and antimetastatic effects against multiple cell lines (15). Soyasaponins, a class of triterpenoids that encompasses three groups of sapogenols designated A, B and E, are comprised of a neutral, non-polar oleane-12-ene triterpene aglycone with differing sugar moieties linked at one or more glycosylation sites (16). Humans are exposed to soyasaponins through the dietary intake of select soy foods, with the B-group being the predominant class (16). Crude soybean saponin mixtures at concentrations ranging from 150 to 2400 p.p.m. are growth inhibitory against multiple human colon adenocarcinoma cell lines in vitro. Treatment of human colon cancer cell lines with soyasaponins suppressed growth, induced morphological alterations, increased multiple markers of differentiation, and inhibited protein kinase C (PKC) activity (17–19). Thus, soy saponins exhibit inhibitory effects.
toward neoplastic cells, actions that are similar to those of various plant saponins reported to be anti-neoplastic and anticarcinogenic agents (15). Following consumption of soy, soyasaponins appear to pass undigested through the small intestine, may be partially metabolized to sapogenols by bacterial glycosidases in the colon and the resulting mixture of saponins and sapogenols can presumably interact with the colonic epithelium (20–22). Soyasaponins could therefore potentially modulate colon carcinogenesis.

Although soyasaponin mixtures can suppress colon cancer multiplication, the bioactivity of specific groups of soyasaponins and their core sapogenols is poorly understood. Therefore, the objective of the present study was to characterize the effects of a soyasaponin fraction containing only the soyasapogenol B glycosides on the proliferation, cell-cycle distribution and programmed cell death (PCD) of the HCT-15 human colon adenocarcinoma cell line.

Materials and methods

Reagents

A purified B-group soyasaponin sample was prepared by Mark Berhow (USDA, Agricultural Research Service, National Center for Agricultural Utilization Research, Peoria, IL) from material provided by Organic Technologies (Coshocton, OH). The proportion of the five different B-group soyasaponins in the sample has been characterized previously using analytical HPLC (23). The saponin storage stock was prepared by adding 500 mg of soyasaponin sample to 1 ml of dimethylsulfoxide (DMSO) and using serial dilutions to produce additional concentrations. A final concentration of 0.1% DMSO was used for experimental tissue culture. Primary antibodies against cyclin-dependent kinase 2 (CDK-2), cyclin A, cyclin B, p27 and actin, and secondary anti-rabbit IgG horseradish peroxidase (HRP) conjugate and anti-mouse IgG HRP conjugate were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); the primary antibody against CDK-2 phosphorylated at tyrosine 15 was purchased from Cell Signaling Technologies (Beverly, MA); and the antibody against microtubule-associated protein light chain 3 (LC-3) was a generous gift from Dr Noboru Mizushima (National Institute for Basic Biology, Okazaki, Japan).

Cell culture

The human colon adenocarcinoma HCT-15 cell line was obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in Eagle’s MEM supplemented with 10% heat-inactivated fetal bovine serum, penicillin (50 U/ml) and streptomycin (50 µg/ml). The non-transformed human colon epithelial NCM460 cell line, a generous gift from Dr Richard Benya (Section of Digestive Disease and Nutrition; University of Illinois at Chicago), was cultured in Ham’s F-12 supplemented with 20% heat-inactivated fetal bovine serum, insulin (0.5 µU/ml), hydrocortisone (0.4 µg/ml) and glutamine (0.58 µg/ml). Cells were maintained at 37°C in a humidified atmosphere of 5% CO2, and were used in subsequent experiments between subculture passages six and ten. Cells were routinely plated at a density of 12 000/cm². On the following day, designated 0 h, media was replaced and either soyasaponin treatment or DMSO vehicle was added. Media and treatments were changed after 24 h of incubation. To obtain synchronous cell populations for cell-cycle analysis and immunoblotting of cell cycle-related proteins, cells were incubated with a final concentration of 1 mM hydroxyurea for 14 h. Cells were released from the block by washing twice with media and were then allowed to grow for 2 h in fresh media. This time point was designated as 0 h, and soyasaponin treatments commenced as described previously.

Proliferation assay

Adherent cells were collected after trypsinization, and following low-speed centrifugation cells were suspended in PBS. Total cells were determined using a hemocytometer with cell viability measured by trypan blue exclusion.

Cell-cycle analysis

Cells were suspended in 0.5 ml PBS, fixed with 70% ice-cold ethanol, and incubated overnight at 4°C. Cells were then lysed in 1 ml of DNA staining solution (0.1 mg/ml ribonuclease type II A, 0.1% NP-40 and 0.25 mg/ml propidium iodide). DNA content was measured using a Coulter EPICS XL-MCL flow cytometer, and EPICS XL System II software was used to generate cellular DNA histograms.

Transmission electron microscopy

For electron microscopic determination of ultrastructural alterations, cells were fixed in ice cold 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, post-fixed in osmium tetroxide, embedded in epoxy Lx112 and 0.1-µm thin sections were stained with uranylacetate/lead citrate. Thin sections were viewed with a Hitachi H600 electron microscope.

MDC incorporation assay

Mature autophagic vacuoles (AVs) were identified based on their AV-specific incorporation of monodansylcadaverine (MDC) (24). For visualization of AVs, cells were incubated with 50 µM MDC for 60 min at 37°C, and then fixed in 4% paraformaldehyde for 15 min according to the method of Biederbick et al. (24). Cells were viewed immediately using the 63 x oil immersion objective of a Zeiss Axiowert 100 fluorescent microscope (356 nm excitation filter and 545 nm barrier filter), and images were obtained with a Roper Scientific Coolsnap fx color camera and processed with MCID software. Intracellular MDC also was quantified using procedures described previously (25). After incubation with 50 µM MDC for 60 min at 37°C, cells were collected in 10 mM Tris-HCl, pH 8, containing 0.1% Triton-X100. MDC incorporation was measured using fluorescence photometry in a Packard Fluorocounter microplate reader (excitation wavelength 380 nm and emission filter 525 nm). MDC values were expressed as specific activity after being normalized to cell number by adding ethidium bromide solution to a final concentration of 0.2 µM and measuring DNA fluorescence (excitation wavelength 530 nm and emission filter 590 nm).

To determine whether 3-methyladenine (3-MA), an inhibitor of macroautophagy (26), reduced AV development, cells were cultured in media containing 10 mM 3-MA, and MDC incorporation was determined as described above.

Immunoblotting

Cells were harvested in lysis buffer [1% sodium deoxycholate, 1% Triton-X100, 0.01% sodium dodecyl sulfate (SDS), 0.15 M NaCl, 0.05 M Tris, 0.0005 M EDTA, 0.05 M NaF, 0.01 M sodium pyrophosphate, 0.0005 M sodium orthovanadate, 0.001 M phenylmethylsulfonyl fluoride, 0.02 mg/ml aprotinin, 0.02 mg/ml leupeptin and 0.02 mg/ml pepstatin in distilled water], and equivalent amounts of protein were subjected to 7.5%–HCl SDS-polyacrylamide gel electrophoresis prior to transfer to a nitrocellulose membrane. Membranes were blocked with blocking buffer for 1 h, and probed with primary antibodies. After treatment with the secondary antibody coupled with HRP, antibody binding was detected by enhanced chemiluminescence. Density for each band was analyzed using the Scion Image Program (NIH, Bethesda, MD). Equal protein loading was confirmed by probing β-actin.

Immunoprecipitation and kinase activity assay

Cells were harvested in NP-40 lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 50 mM NaF, 1 mM sodium orthovanadate, 0.5% NP-40, 0.001 M phenylmethylsulfonyl fluoride, 0.02 mg/ml aprotinin, 0.02 mg/ml leupeptin and 0.02 mg/ml pepstatin). 200 µg of lysate proteins were immunoprecipitated overnight with 2 µg of CDK-2 antibodies (Santa Cruz, CA) along with protein A-agarose beads in NP-40 lysis buffer. The immunoprecipitates were washed three times in lysis buffer, washed once in kinase assay buffer (20 mM HEPES pH 7.5, 20 mM MgCl2), and re-suspended in kinase assay buffer supplemented with 10 µM ATP, 0.5 mM EDTA, 0.2 mM sodium orthovanadate and 1 mM DTT. Ten micrograms of histone type III-S and 5 µCi of [γ-32P]ATP (7000 Ci/mmol) were added to each sample to begin the kinase reaction. Mixtures were incubated for 1 h on ice, and stopped with the addition of equal amounts of 2× SDS-PAGE sample buffer. Samples were boiled for 3 min, and then separated using 10% Tris–HCl SDS-polyacrylamide gel electrophoresis prior to transfer to a nitrocellulose membrane. Reaction products were detected by autoradiography and densitometry was performed as described above.

Statistical analysis

Unless otherwise noted, experiments were performed in triplicate, and were repeated at least twice. Statistical differences between control and treated samples were determined by the Student’s t-test using SAS software. P values of <0.05 were considered significant.

Results

B-group soyasaponins selectively inhibit proliferation of HCT-15 cells

Treatment of asynchronous cultures of HCT-15 cells with B-group soyasaponins decreased cell proliferation in a time- and concentration-dependent manner (Figure 1A). As early as 24 h after initiation of soyasaponin treatment viable cell numbers were significantly reduced by 31–54%, compared
with controls, at concentrations of 25–500 p.p.m., and an IC$_{50}$ was identified as ~100 p.p.m. Similarly, after 48 h of soya-saponin treatment viable cell numbers were significantly reduced by 45–68%, compared with controls. A significant increase in non-viable HCT-15 cell numbers was observed after 48 h exposure to saponin concentrations >100 p.p.m. (Figure 1C). The percentage of cells that absorbed trypan blue dye increased from 5% in control samples to 10–15% in treated cells, indicating that cell death may contribute to the reduction in viable cell number.

**B-group soyasaponins induce a transient S-phase accumulation of HCT-15 cells**

Treatment of synchronous HCT-15 cells with 25 and 100 p.p.m. B-group soyasaponins resulted in a significant accumulation of cells in S-phase as early as 6 h post-treatment (Figure 2B). Soyasaponin treatment of 100 p.p.m. increased the fraction of cells in S-phase by an average of 50%, compared with controls, after 6–24 h of treatment, and similar results were observed following treatment with 25 p.p.m. soyasaponins. A significant reduction of the cell fraction in G$_{2}$/M was also observed at 24 and 48 h post-treatment at both soyasaponin concentrations, compared with controls (Figure 2C). Accumulation of cells in S-phase was associated with inhibition of CDK-2 activity, the predominant S-phase kinase (Figure 3A and B). The activity of CDK-2 was decreased significantly by ~20%, compared with controls, following treatment with 100 p.p.m. B-group soyasaponins for 6 and 12 h. Concentrations of cell cycle-related proteins varied in a time-dependent manner (Figure 3C and D). Although total CDK-2 protein levels did not change at the times measured, a significant 1.9-fold increase in the inhibitory phosphorylation of CDK-2 at tyrosine-15 was detected at 12 h post-treatment, compared with controls. Cyclin A levels increased by a significant 50% at 6 h post-treatment with 100 p.p.m. soyasaponins. Associated with the reduction of cells in G$_{2}$/M there was a significant 33% decrease in protein concentrations of cyclin B at 12 h post-treatment that returned to control levels by 24 h post-treatment. In addition, treatment with 100 p.p.m. soyasaponins for 48 h significantly increased the protein content of the cyclin-dependent kinase inhibitor (CDKI) p27$^{\text{CIP1/KIP1}}$ by 1.8-fold, compared with controls, although no alterations were observed in concentrations of this protein at earlier time points. Finally, the ratios of protein concentrations of p27$^{\text{CIP1/KIP1}}$ to CDK-2 were calculated to be 1.4 and 2.1 by 24 and 48 h post-treatment, respectively.

**B-group soyasaponins stimulate macroautophagy in HCT-15 cells**

In order to characterize the morphological changes in HCT-15 cells following 48 h of exposure to 100 p.p.m. B-group soyasaponins, ultrastructural details were determined using transmission electron microscopy (TEM). We observed two morphologically distinct cell responses based on microscopic features of macroautophagy reported previously (27). Therefore, cell characteristics were assigned into two categories (Figure 4A and B) so that soyasaponin-induced changes could be more accurately quantified (Figure 4C). Based on these criteria, we determined that B-group soyasaponin treatment significantly decreased the percentage of cells exhibiting characteristics of type A cells (~2.4-fold), the predominant cell type in control cell populations. In addition, there was a significant increase in the proportion of cells exhibiting the type B autophagic morphology (~4.5-fold).

To determine whether a portion of the numerous vacuoles observed with TEM were AV$_{d}$, MDC incorporation was evaluated. It has been determined previously that the incorporation of MDC into vacuoles is a definitive characteristic of AV$_{d}$

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**Fig. 1.** B-group soyasaponins decrease the number of viable HCT-15 cells (A) while the number of non-transformed NCM460 cells is unchanged (B). (C) B-group soyasaponins increase the percentage of non-viable HCT-15 cells by 48 h post-treatment. Cells were counted on a hemocytometer, and viable cells were identified by trypan blue exclusion. Values represent means ± SD ($n=3$) for each treatment, and are representative of three independent experiments. *Differs significantly from control at $P<0.05$. **Fig. 2.** B-group triterpenoid soyasaponins induce autophagy in HCT-15 cells **Fig. 3.** B-group triterpenoid soyasaponins induce autophagy in HCT-15 cells **Fig. 4.** B-group triterpenoid soyasaponins induce autophagy in HCT-15 cells **Fig. 5.** B-group triterpenoid soyasaponins induce autophagy in HCT-15 cells
Visualization of cellular MDC incorporation revealed that AVd were abundant in treated cells but nearly absent in control cells (Figure 5A and B). MDC incorporation was increased significantly by exposure to both 25 and 100 p.p.m. B-group soyasaponins, with specific activity averaging 8% in control cells and 27–35% in cells treated with 100 p.p.m. soyasaponins (Figure 5C). The most significant effect, a 1.8- to 3.3-fold increase in MDC incorporation compared with controls, was observed after 6–48 h exposure to 100 p.p.m. B-group soyasaponins. Concurrent treatment for 48 h of HCT-15 cells with 100 p.p.m. B-group soyasaponins and 3-MA, a known inhibitor of macroautophagy (26), reduced MDC incorporation to control levels.

It has been determined that the bulk of the proteins requisite for the autophagic pathway are constitutively expressed. However, it has been recognized that both de novo transcription of LC-3 I, a microtubule associated protein, and its subsequent post-translational modification to the membrane bound LC-3 II are requisite for the initial formation of immature...
AV (AVᵢ), the precursor to the AVᵣ (28). Therefore, we measured the concentrations of LC-3 to determine whether the morphological evidence of macroautophagy was associated with molecular changes characteristic of induction of macroautophagy (Figure 6). We found that concentrations of LC-3 I protein were significantly elevated by 4.25-fold at 6 h post-treatment with 100 p.p.m. B-group soyasaponins, and they remained significantly elevated through 48 h of exposure to soyasaponins. Concentrations of the activated LC-3 II protein were also significantly elevated by 3-fold at 6 h post-soyasaponin treatment, and they remained significantly elevated through 24 h post-treatment.

Discussion

The present studies demonstrate that purified B-group soyasaponins inhibited the proliferation of the HCT-15 colon adenocarcinoma cell line and induced macroautophagy in a dose- and time-dependent manner. This is the first report characterizing this novel mechanism by which B-group soyasaponins exert their anticancer activity, and it provides a new means by which soyasaponins may modulate colon carcinogenesis in vitro and potentially in vivo.

We observed that B-group soyasaponin treatment resulted in a time- and concentration-dependent inhibition of HCT-15 cell multiplication. The largest reduction, 68% fewer cells compared with the control, was observed after 48 h of exposure to 500 p.p.m. B-group soyasaponins. The growth inhibition we observed is consistent with results reported previously for inhibition of human colon adenocarcinoma cell proliferation by a crude soyasaponin extract (17,19). However, in the present studies growth was significantly suppressed at concentrations much lower than those used in the latter studies. This increased anticancer potency may in part be due to the purified B-group soyasaponins we used for the experiments. In support

Fig. 4. Ultrastructural details of HCT-15 cells treated with 100 p.p.m. B-group soyasaponins for 48 h. (A) Representative cells viewed using TEM at 5000×. Bar represents 2 μm. (a) Control HCT-15 cell classified as A. (b) Treated HCT-15 cell classified as B. Note the absence of mitochondria, large vacuoles containing degraded cellular material (black arrows) and residual bodies (white arrows). (B) Description of the morphological criteria used to quantify cell types. (C) Quantification of cell types. Values are mean ± SD of the percentage of cells in each morphological group. A minimum of 100 cells were analyzed per duplicate sample. *Statistically significant difference compared with control at P < 0.05.
of this proposition, treatment of the MCF-7 and MDA-MB-231 human breast cancer cell lines with B-group soyasapogenols resulted in a greater growth inhibition compared with cells treated with A-group soyasapogenols (29). Of interest, exposure to B-group soyasaponins did not alter proliferation of the non-transformed human colon epithelial NCM460 cell line, suggesting that the saponin mixture may selectively inhibit the growth of cancerous cells.

There are multiple mechanisms by which saponins might exert their anticancer actions. For example, small quantities of B-group soyasaponins may be absorbed by colon cancer cells through a saturable uptake system (30). Alternatively, it has been hypothesized that soyasaponins may affect membrane integrity by either binding to or inserting into the plasma membrane (31). We observed that B-group soyasaponin treatment resulted in only a 5–10% decrease in HCT-15 cell viability at concentrations ≤100 p.p.m., which suggests that the effects of the soyasaponins in our studies are not simply due to their membrane disruptive actions. The effects on membrane stability are probably dependent upon both saponin concentration and the chemical properties of the aglycone. Previous reports indicated a comparable 10% decrease in cell viability following crude soyasaponin treatment at 150 p.p.m., while treatment concentrations of 600 p.p.m. resulted in a >40% decrease in cell viability (17), a finding that may be explained by the higher concentrations of soyasaponins utilized. Regarding the aglycone form, those with acidic groups have a larger impact on membrane disruption than neutral aglycones. Soyasaponins are composed of neutral aglycones, which may partly contribute to the lack of immediate membrane destabilization.

Saponins may also suppress carcinogenesis by interfering with cell-cycle progression. In this regard, we observed that
B-group soyasaponin treatment resulted in a time-dependent accumulation of cells in S-phase. To our knowledge this is the first report of an S-phase delay induced by saponins. In contrast to our findings, human glioma cells were arrested in G0/G1 following B-group soyasaponin treatment (32). In addition, other saponins, primarily ginsenosides, induced a cell type-specific G0/G1 arrest (15). Thus, cell-cycle alterations induced by saponin treatment may be dependent upon cell type and saponin source.

The modest accumulation of cells in S-phase that we detected appears in part to be mediated through inhibition of CDK-2 activity, as we observed a ~20% reduction of the kinase activity associated with saponin treatment. Successful progression of cells through S-phase and into G2 is dependent upon sustained activation of CDK-2, with decreased CDK-2 activity being reported to precipitate an S-phase accumulation (33). The significant rise in concentrations of cyclin A by 6 h post-treatment, consistent with the increased fraction of cells in S-phase, suggests that cyclin A is not a rate-limiting factor for CDK-2 activation. A model for the phosphorylation/dephosphorylation events leading to CDK-2 activation has recently been proposed (34). According to this model CDK-2 is first phosphorylated at the inhibitory tyrosine-15 site. Following the binding of cyclin A, CDK-2 is next activated by phosphorylation at threonine-160. Finally, CDK-2 is dephosphorylated at the inhibitory tyrosine-15 residue, resulting in an active CDK-2 enzyme. We observed a significant increase in inhibitory phosphorylation at tyrosine-15 by 12 h post-treatment, a finding that suggests that reduced CDK-2 activity is mediated in part through maintenance of the inhibitory phosphorylation. Furthermore, the significant 12 h post-treatment decrease in protein expression of cyclin B is indicative of reduced CDK-2 activity (35), and may contribute to the reduced G2/M cell fraction. Finally, we observed a significant 1.8-fold increase in the CDKI p27\textsuperscript{kip1}/cip1 at 48 h post-treatment. This CDKI, when localized to the nucleus, can block CDK-2 activity by inhibiting its association with cyclin A (36), an effect observed when the p27\textsuperscript{kip1}/cip1/CDK ratio is >1 (37). We calculated this ratio to be 1.4 by 24 h post-treatment, and 2.1 at 48 h post-treatment. More studies are needed in the future to clarify the protein interactions that contribute to S-phase delay, as well as to delineate the role of upstream regulatory events.

In our view the most significant finding of the present studies is that treatment of cells with B-group soyasaponins did not induce overt apoptosis but rather induced morphological alterations characteristic of macroautophagy, a cellular process that has recently received increased scrutiny because of its role in maintaining cell homeostasis as well as contributing to Type II PCD (27,38,39). In fact, there are recent suggestions that defective macroautophagy may be a contributor to carcinogenesis (40). The morphological alterations that we observed, such as reduced cytoplasmic density and vacuolization, are similar to those reported by others after 48 h of treatment with 2400 p.p.m. crude soyasaponins (18). Likewise, exposure to o-hederin, a triterpenoid saponin with an oleanolic acid aglycone, was reported to induce similar morphological alterations in B16 melanoma cells (41). We confirmed that a large fraction of the vacuoles abundant in treated cells were autophagic (as opposed to endocytic) by demonstrating sustained vacuolar incorporation of MDC, a specific marker of AV\textsubscript{3} (24). We also established that treatment with 3-MA, a specific inhibitor of macroautophagy (26), blocked MDC accumulation in HCT-15 cells treated with B-group soyasaponins. An up-regulation of macroautophagy was further supported by a time-dependent increase in both LC-3 I and the activated LC-3 II proteins essential to the formation of AV (28). Thus, we have documented several morphologic and molecular changes that strongly suggest that B-group soyasaponins induce macroautophagy in the HCT-15 cell line. Whether these saponins induce similar changes in other human colon cancer cell lines warrants further study.

This capacity of B-group soyasaponins to induce macroautophagy is noteworthy, since macroautophagy is down regulated in cancers (42). However, this finding must be interpreted with caution as induction of macroautophagy can promote both tumor progression and cancer cell death (43). Under stressful conditions including nutrient deprivation, hypoxia and both radiation and chemotherapies, macroautophagy can serve as a self-defensive adaptation by recycling essential molecules, and by sequestering toxic agents thereby reducing apoptotic triggers (43,44). Thus, it is possible that the B-group soyasaponin treatment induces macroautophagy as a protective mechanism in HCT-15 cells. Conversely, macroautophagy is becoming recognized as an important contributor to Type II PCD through the selective lysosomal degradation of cytoplasm and organelles. Prolonged periods of macroautophagy give rise to Type II PCD, a caspase-independent series of events characterized by the formation of numerous AV followed by cytoplasmic and, in some instances, nuclear destruction (27,38,39). Therefore, it is important to identify compounds that can up-regulate and, more importantly, sustain this process. It is noteworthy that an induction of macroautophagy has been reported in MCF-7 cells after exposure to tamoxifen (45). In addition, arsenic trioxide stimulates remission in patients with malignant gliomas through induction of macroautophagy (46). Therefore, compounds that induce macroautophagy have demonstrated the potential to be effective chemotherapeutic agents in clinical trials.

The B-group soyasaponin-induced alterations in cell-cycle distribution that we observed also have been associated with macroautophagy. Although a G1 cell-cycle arrest is frequently observed in conjunction with macroautophagy (43), S-phase accumulation and morphological indications of macroautophagy have been observed in prostate cancer cells following treatment with prostaglandin J\textsubscript{2} (47) as well as in ovarian cancer cells treated with resveratrol (48). Of interest, exogenous induction of p27\textsuperscript{kip1}/cip1 has been associated with the appearance of morphological autophagic features in malignant glioma cells (49).

In summary, we report that B-group soyasaponins inhibited the proliferation of HCT-15 cells, a phenomena associated with S-phase accumulation and induction of macroautophagy, the predominant characteristic of Type II PCD. Diverse signaling pathways have been implicated in the control of mammalian macroautophagy as reviewed by Petiot et al. (50). Future research is warranted to determine the molecular events initiated by B-group soyasaponins that result in the aforementioned findings, as well as the mechanism through which soyasaponins interact with colon cancer cells. In addition, a study of animals fed a diet rich in B-group soyasaponins should be conducted to evaluate whether the macroautophagy observed in vitro is also relevant in vivo. Our findings have public health significance, as it
has been estimated that daily consumption of 12.5 g of soy flour could result in B-group saponin concentrations of ~100 p.p.m. in the ascending colon (15). Concentrations would probably increase as fecal matter amasses during transit through the sigmoid colon, a major site of colon cancer prevalence. Therefore, the IC50 value of 100 p.p.m. B-group soyasaponin reported in this study is physiologically relevant and suggests potential usefulness in colon cancer chemoprevention strategies.

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