Silymarin induces apoptosis primarily through a p53-dependent pathway involving Bcl-2/Bax, cytochrome c release, and caspase activation

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Abstract
Silymarin, a plant flavonoid, has been shown to inhibit skin carcinogenesis in mice. However, the mechanism responsible for the anti-skin carcinogenic effects of silymarin is not clearly understood. Here, we report that treatment of JB6 C141 cells (preneoplastic epidermal keratinocytes) and p53+/+ fibroblasts with silymarin and silibinin (a major constituent of silymarin) resulted in a dose-dependent inhibition of cell viability and induction of apoptosis in an identical manner. Silymarin-induced apoptosis was determined by fluorescence staining (8–64% apoptosis) and flow cytometry (12–76% apoptosis). The silymarin-induced apoptosis was primarily p53 dependent because apoptosis occurred to a much greater extent in the cells expressing wild-type p53 (p53+/+, 9–61%) than in p53-deficient cells (p53−/−, 6–20%). The induction of apoptosis in JB6 C141 cells was associated with increased expression of the tumor suppressor protein, p53, and its phosphorylation at Ser15. The constitutive expression of antiapoptotic proteins Bcl-2 and Bcl-xl were decreased after silymarin treatment, whereas the expression of the proapoptotic protein Bax was increased. There was a shift in Bax/Bcl-2 ratio in favor of apoptotic signal in silymarin-treated cells, which resulted in increased levels of cytochrome c release, apoptotic protease-activating factor-1, and cleaved caspase-3 and poly(ADP-ribose) polymerase in JB6 C141 cells. The shift in Bax/Bcl-2 ratio was more prominent in p53+/+ fibroblasts than in p53−/− cells. Silymarin-induced apoptosis was blocked by the caspase inhibitor (Z-VAD-FMK) in JB6 C141 cells, which suggested the role of caspase activation in the induction of apoptosis. These observations show that silymarin-induced apoptosis is primarily p53 dependent and mediated through the activation of caspase-3. [Mol Cancer Ther 2005;4(2):207–16]

Introduction
Silymarin, a flavonoid, is isolated from the fruits and seeds of the milk thistle (Silybum marianum L. Gaertn.). Silymarin is composed of mainly silibinin (~90%) with small amounts of other silibinin stereoisomers (i.e., isosilybin, dihydroisilybin, silydianin, and silychristin; ref. 1). Silymarin has been shown to have anti-inflammatory, anti-oxidative, and anticarcinogenic properties in in vitro animal models (2). Because of these properties, silymarin has been tested in various in vivo and in vitro models to test its efficacy against cancer and most specifically against the prevention of skin carcinogenesis. In earlier studies, we have shown that topical treatment of silymarin to sensitive-to-carcinogen mice resulted in inhibition of 7,12-dimethylbenz(a)anthracene–initiated and 12-O-tetradecanoylphorbol-13-acetate–promoted skin tumorigenesis in terms of tumor incidence, tumor multiplicity, and tumor growth (3). Besides its protection against chemical carcinogenesis, Katiyar et al. (4) have shown that topical treatment of silymarin to SKH-1 hairless mice inhibited solar UV radiation–induced skin tumorigenesis or photocarcinogenesis. In further studies, Katiyar (5) has shown that treatment of silymarin to SKH-1 hairless mouse skin inhibits UV-induced markers of oxidative stress. The chemopreventive studies conducted against both chemical carcinogenesis and photocarcinogenesis clearly revealed that silymarin specifically possesses strong inhibitory effect on tumor promotion stage of multistage carcinogenesis (2–4).

It has long been recognized that tumor suppressor gene p53 is induced by DNA damage (6). The resulting increases in p53 lead either to the induction of cell cycle arrest or apoptosis (7, 8). Thus, p53 activation contributes to suppression of malignant transformation, in other words, functional p53 provides a protective effect against tumor growth. Therefore, apoptosis has been characterized as a fundamental cellular activity occurring under a wide range of physiologic and pathologic conditions (9–11). In UV carcinogenesis, silibinin, a major and active component of silymarin, has been shown to up-regulate the expression of p53 protein in mouse skin (12). Yoo et al. (13) have shown that treatment of silibinin to human endothelial ECV304 cells induced apoptosis via modulation...
Silymarin induces apoptosis via p53-dependent pathway

Materials and Methods

Chemicals and Antibodies

Silymarin and silibinin were purchased from Sigma Chemical Co. (St Louis, MO). Hoechst 33342 and Annexin V–conjugated Alexa Fluor 488 Apoptosis Detection Kit were purchased from Molecular Probes, Inc. (Eugene, OR). Antibodies for p53, Bcl-2, cytochrome c, apoptotic protease-activating factor-1 (Apaf-1), and -actin were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), whereas antibodies for phospho-p53 (p53 at Ser15), Bcl-xl, Bax, and cleaved caspase-3 (specific for 17 and 19 kDa) were procured from Cell Signaling Technology, Inc. (Beverley, MA), and antibodies for PARP was obtained from Upstate Cell Signaling Solutions (Charlottesville, VA). The caspase inhibitor Z-VAD-FMK was purchased from R&D Systems, Inc. (Minneapolis, MN). DMEM, penicillin, streptomycin, fetal bovine serum (FBS), and trypsin/EDTA were purchased from CellGro (Herndon, VA). Protein assay kit were procured from Bio-Rad (Hercules, CA). Enhanced chemiluminescence detection kit for Western blotting was purchased from Amersham Pharmacia Biotech (Piscataway, NJ).

Cell Lines and Culture Conditions

Preneoplastic epidermal cell line JB6 C141 was cultured in monolayers with DMEM supplemented with 10% (v/v) heat-inactivated FBS and glutamine (2 mmol/L) at 37°C in a humidified atmosphere of 5% CO2. p53 wild-type (p53+/+) and p53-deficient (p53−/−) mouse embryonic fibroblast cells were cultured in DMEM supplemented with 15% FBS and glutamine (2 mmol/L) at 37°C in a humidified atmosphere of 5% CO2. For analysis of apoptosis, JB6 C141 cells, p53+/+, and p53−/− fibroblasts were serum starved in 0.5% FBS/DMEM overnight and thereafter treated with silymarin for 24 hours.

Cell Viability Assay

The effect of silymarin and silibinin on the cellular proliferation and viability of JB6 C141, p53+/+, and p53−/− cells was determined by 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) assay as described previously (21, 22). Briefly, ~1 × 104 cells were plated in 96-well culture plates and treated with varying concentrations of silymarin and silibinin (0–80 μg/mL) in fresh media for 12, 24, and 48 hours. At the desired time points after silymarin or silibinin treatment, medium was aspirated and MTT (50 μL, 5 mg/mL in PBS) was added in each well. Incubation was continued for additional 2 hours at 37°C. Thereafter, the plates were centrifuged and purple colored precipitates of formazan were dissolved in 150 μL of dimethyl sulfoxide. The color absorbance was recorded at 540 nm with a Bio-Rad 3350 microplate reader with a reference at 650 nm serving as a blank. The reduction in cell viability after silymarin or silibinin treatment was expressed in terms of percent of control (nonsilymarin or nonsilibinin treated) cells.

Detection of Apoptotic Cells by Fluorescence Staining

Cells were incubated with silymarin (0–80 μg/mL) for 24 hours and were then washed with PBS buffer, thereafter, fixed in freshly prepared ice-cold paraformaldehyde (0.1%) for 10 minutes. The cells were then washed with PBS and stained with Hoechst 33342 (50 μg/mL) for 1 minute in the dark. Morphologic changes in cells were observed under fluorescent microscope. The percentage of apoptotic cells was determined after counting at least 200 cells per treatment group. The experiment was repeated thrice.

Quantitative Analysis of Apoptotic Cells by Flow Cytometry

Quantitative analysis of apoptotic cells with or without treatment of silymarin was done by using the Annexin V–conjugated Alexa Fluor 488 Apoptosis Detection Kit following the manufacturer’s protocol, as was done previously (22). Briefly, after overnight serum starvation, JB6 C141 cells and p53+/+ or p53−/− fibroblasts were treated with silymarin (0–80 μg/mL) for 24 hours. Cells were harvested, washed with cold PBS, and subjected to Alexa 488 and propidium iodide staining in binding buffer at room temperature for 15 minutes in the dark. Apoptotic cells, stained with Alexa 488 and propidium iodide, were analyzed by fluorescence activated cell sorting (FACSCalibur, BD Biosciences, San Jose, CA) using...
CellQuest 3.3 software. The apoptotic cells stained with Alexa 488 showed green fluorescence, whereas the cells stained with both Alexa 488 and propidium iodide showed red and green fluorescence.

**Immunoprecipitation, Immunoblotting, and Western Blotting**

JB6 C141 cells and p53+/+ or p53−/− fibroblasts were incubated with silymarin (0–80 μg/mL) for 24 hours. The cells were harvested, washed with cold PBS [10 mmol/L (pH 7.4)], and lysed with ice-cold lysis buffer [50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L EGTA, mmol/L EDTA, 1% NP40, 1 mmol/L sodium orthovanadate, 20 mmol/L NaF, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, and 10 μg/mL leupeptin (pH 7.4)] for half an hour over ice, as described previously (23). The lysates were centrifuged at 14,000 rpm for 10 minutes at 4°C and the supernatants were used for Western blot analysis. The lysates were also immunoprecipitated using p53 antibodies and protein G plus/protein A-agarose. The beads were washed thrice with PBS, and were examined for phosphorylated p53 protein specific to Ser15. The protein concentration was determined by the DC Bio-Rad assay using the manufacturer’s protocol. For Western blotting, proteins (30–50 μg) were resolved on 10% to 12% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane. The membranes were incubated with the appropriate concentrations of primary antibodies in blocking buffer overnight at 4°C, as described previously (23). After washing in PBS, the membrane was incubated with the secondary antibody conjugated with horseradish peroxidase at room temperature for 1 hour. Protein bands were visualized using the enhanced chemiluminescence detection system (Amersham Life Science, Inc., Piscataway, NJ). The blots were stripped and reprobed for β-actin antibody to verify equal protein loading and equal transfer of proteins from gel to membrane. The relative intensity of each protein band from different treatment groups was determined using computerized OPTIMAS 6.2 software program.

**Statistical Analysis**

The Student’s t test was done to determine the statistical significance of the difference in the absolute values of the apoptotic cell death between the silymarin-treated and nonsilymarin-treated groups. The silymarin-induced apoptosis in experimental cell lines was considered significant at P < 0.05 where not mentioned.

**Results**

**Both Silymarin and Silibinin Decrease Cell Viability of JB6 C141 Cells**

A preliminary screening was done to assess the effect of silymarin and silibinin on cellular proliferation and viability of JB6 C141 cells at different time points using the MTT assay. Also, we were interested to confirm if there was a significant difference in the absolute values of silymarin and silibinin in terms of their chemopreventive potential. As shown in Fig. 1, there was a dose-dependent and time-dependent reduction in the viability of JB6 C141 cells treated with silymarin or silibinin (20–80 μg/mL). Treatment of JB6 C141 cells with silymarin for 12 hours did not result in a significant reduction in cell viability even at a highest dose of silymarin used (80 μg/mL), whereas treatment of silibinin (40–80 μg/mL) for 24 hours resulted in a significant reduction in cell viability (35–74%, P < 0.01–0.001). The cell viability was further decreased when cells were incubated with silymarin for 48 hours (49–83%, P < 0.01–0.001). Similar results were also observed with the treatment of silibinin to JB6 C141 cells under identical experimental conditions (data not shown). When reduction in cell viability data was compared between silymarin and silibinin at the equal treatment doses and at the same time points, it was found that the difference was not more than ±6% (data not shown), and this difference was not statistically significant. Because there was no significant difference in between silymarin and silibinin in terms of their cytotoxic effects on JB6 C141 cells, silymarin was selected as a potent candidate for further in vitro studies. Additionally, because the cell viability of JB6 C141 cells was significantly decreased by silymarin after its treatment for 24 hours, this time point was selected for all further mechanistic studies.

**Silymarin Induces Apoptosis in JB6 C141 Cells**

To determine whether decrease in cell viability after silymarin treatment is the result of apoptosis in JB6 C141 cells, fluorescence staining was done to detect the apoptotic cells following the procedure of He et al. (19). Treatment of JB6 C141 cells with silymarin at the doses of 20, 40, 60, and 80 μg/mL resulted in a dose-dependent increase in number of apoptotic cells, which ranged from 8% to 64% (Fig. 2A). Silymarin-induced apoptosis in JB6 C141 cells was further quantitatively analyzed by flow cytometry (Fig. 2B). For this purpose, cells were stained with Alexa 488 and propidium iodide. The cells in the bottom right
Silymarin Induces Apoptosis via p53-Dependent Pathway

Figure 2. A, silymarin induces apoptosis in JB6 C141 cells. Apoptosis was determined by fluorescence staining as detailed in Materials and Methods. Briefly, cells were treated with silymarin for 24 h; thereafter, morphologic changes in JB6 C141 cells caused by apoptosis were observed under fluorescence microscope. Cells were treated without silymarin (a) and with silymarin (b, c, d, and e) for 20, 40, 60, and 80 µg/mL, respectively, for 24 h after overnight serum starvation. The experiment was repeated twice. Columns, average % apoptotic cells for each treatment group; bars, ± SD (f). At least 200 cells were counted in a blinded manner to score the % apoptosis in each treatment group. *, P < 0.05 versus control (nonsilymarin); **, P < 0.001 versus control (nonsilymarin). B, silymarin-induced apoptosis in JB6 C141 cells was determined by flow cytometry using Annexin V-Alexa Fluor 488 (Alexa488) Apoptosis Vybrant Assay kit. Cells were incubated with Alexa488 and propidium iodide in binding buffer at room temperature following the manufacturer’s protocol. a, control cells (nonsilymarin treatment); b, c, d, and e, treated with 20, 40, 60, and 80 µg/mL, respectively, of silymarin for 24 h after overnight serum starvation as detailed in Materials and Methods. Bottom right quadrant (LR), % early apoptotic cells (Alexa488-stained cells); top right quadrant (UR), % late apoptotic cells (Alexa488 + propidium iodide – stained cells).

quadrant of the histogram (Fig. 2B) represent the number of early apoptotic cells and were stained with Alexa 488. Alexa 488 detects apoptotic cells with externalized phosphatidyl-serine leaflets. The top right quadrant (Fig. 2B) represents the late apoptotic cells and are stained with both Alexa 488 and propidium iodide (19, 22). Treatment of JB6 C141 cells with silymarin (20–80 µg/mL) for 24 hours resulted in increased number of early apoptotic cells which ranged from 10.1% to 75.9% in a dose-dependent manner compared with only 4.5% apoptotic cells in the nonsilymarin-treated group. The total percentage of apoptotic cells (top right + bottom right quadrants) after silymarin treatment (20–80 µg/mL) was dose-dependently increased by 12.3% to 76.2%, whereas only 5.3% apoptotic cells were found in nonsilymarin-treated control cells.

Silymarin Increases p53 Expression and the Phosphorylation of p53 in JB6 C141 Cells

Tumor suppressor protein p53 plays a crucial role in the suppression of transformation, and thus functional p53 provides a protective effect against tumor growth (24). The function of activated p53 is critically dependent on the phosphorylation of p53 protein at the Ser15 residue (25). To determine the possible role of p53 in induction of apoptosis by silymarin in JB6 C141 cells, we assessed the expression of total p53, and phosphorylated p53 at Ser15 by Western blot analysis. The treatment of silymarin (20–80 µg/mL) resulted in a dose-dependent increase in the basal level of p53 protein (Fig. 3, top) as well as increase in its phosphorylation at Ser15 (Fig. 3, bottom). The resultant increase in p53 after silymarin treatment may be because of its stability due to increase in its half-life.

Both Silymarin and Silibinin Decrease Cell Viability in Wild-type p53 Cells (p53+/+) but not in p53-Deficient Cells (p53−/−)

Similar to JB6 C141 cells which express wild-type p53, the effect of silymarin and silibinin was determined on cell viability of p53+/+ and p53−/− fibroblasts using MTT assay (21, 22). Our aim was to determine whether the cytotoxic effect of silymarin or silibinin was associated with the expression of wild-type p53. It was observed that treatment of both p53+/+ and p53−/− fibroblasts with silymarin did not result in significant reduction in cell viability within 12 hours, as was also observed in JB6 C141 cells. However, treatment of p53+/+ cells with silymarin at the doses of 20, 40, 60, and 80 µg/mL for 24 hours resulted in a dose-dependent inhibition of cell viability (10 ± 3% to 68 ± 7%) compared with nonsilymarin-treated cells. Similar to JB6 C141 cells, cell viability of p53−/− cells was further reduced after 48 hours of silymarin treatment (12–74%). In contrast, the inhibition of cell viability in p53-deficient (p53−/−) cells after 12, 24, and 48 hours of silymarin treatment (20–80 µg/mL) was in the range of 5% to 19%. Similar effects were also observed with silibinin treatment under identical experimental conditions (data not shown). Thus, it seemed that p53-deficient cells were resistant to silymarin and silibinin. In other words, the inhibition of cell viability by silymarin and silibinin required, at least in part, p53 protein expression.

Silymarin Induces Apoptosis in p53+/+ Cells but not in p53-Deficient (p53−/−) Cells

Because silymarin and silibinin showed identical effects on reduction of cell viability in p53+/+ and p53−/− cells,
in further studies only silymarin was used. We were interested to determine whether tumor suppressor protein p53 is required for induction of apoptosis caused by silymarin, we determined the effect of silymarin on fibroblast cell lines derived from p53-deficient (p53−/−) mouse embryos and their wild-type p53+/+ counterpart. First, silymarin-induced apoptosis was determined by fluorescence staining, and it was observed that treatment of p53+/+ (wild type) cells with increasing doses of silymarin exhibited a dose-dependent increase in the number of apoptotic cells which ranged from 8-55% (Fig. 4B–E and results are summarized in F) compared with 7% in nonsilymarin-treated cells (Fig. 4A). Quantitative analysis of apoptotic cells was further done by flow cytometry. As shown in Fig. 5, the number of silymarin-induced apoptotic cells during early phase ranged from 6.8% to 48.2% after 24 hours of treatment compared with only 4.0% in the nonsilymarin-treated group. Similarly, silymarin-induced dose-dependent increase in the number of apoptotic cells during the late phase of apoptosis (top right quadrant) which was ranged from 2.7% to 12.8%. Thus, the total number of apoptotic cells after silymarin treatment was 9.5% to 61.0% compared with 7.7% in the nonsilymarin-treated group. These observations indicated that p53+/+ fibroblasts responded in a manner that was similar to JB6 C141 cells.

Figure 3. Silymarin increases the level of p53 and induces phosphorylation of p53 at Ser15 in JB6 C141 cells. Overnight serum starved JB6 C141 cells were treated with varying doses of silymarin (20 –80 μg/mL) for 24 h. Cell lysates were prepared and used for Western blotting of p53, and 200 μg of protein were used for immunoprecipitation to detect the phosphorylation of p53 at Ser15 using a specific antibody, as described in Materials and Methods. Below the blots, relative intensity of the bands was determined. Columns, mean of relative intensity from three experiments; bars, ±SD. *, P < 0.05 versus control (nonsilymarin); **, P < 0.001 versus control.

Figure 4. Silymarin induces a significantly higher number of apoptotic cells in p53 wild-type (p53+/+) fibroblasts compared with p53-deficient (p53−/−) fibroblasts, as determined by fluorescence staining. Both p53+/+ and p53−/− fibroblasts were cultured under identical conditions as was done in JB6 C141 cells except that the fibroblasts were treated with 15% FBS in DMEM. Cells were starved in 0.5% FBS/DMEM overnight and thereafter treated with silymarin in serum containing media for another 24 h. Morphologic changes in p53+/+ and p53−/− fibroblasts undergoing apoptosis were observed under fluorescence microscope. A and G, cells were not treated with silymarin (controls); B–E and H–K, increasing concentrations of silymarin were added to cultures for 24 h by 20, 40, 60, and 80 μg/mL, respectively. F, summarized % apoptotic cells from A–E, L, summarized % apoptotic cells from G–K. The experiment was repeated at least twice. Columns, average % apoptotic cells from each treatment group; bars, ±SD. At least 200 cells were counted in a blinded manner to score the % apoptosis in each treatment group. *, P < 0.05 versus control (nonsilymarin); **, P < 0.005 versus control (nonsilymarin).
Simultaneously, we also evaluated the effect of silymarin on p53-deficient (p53⁻/⁻) fibroblasts. As shown in Fig. 4 (right), the treatment of p53⁻/⁻ fibroblasts with silymarin did not induce apoptosis at the same level as was induced in p53 wild-type fibroblast (p53⁺/⁺; Fig. 4, left). However, treatment of p53⁻/⁻ fibroblasts with silymarin-induced the apoptosis which ranged from 6% to 18% as analyzed by fluorescence staining (Fig. 4H–K and results are summarized in L). The apoptosis in p53-deficient cells by silymarin was further confirmed by flow cytometry (Fig. 5F–J). The percentage of apoptotic cells during early phase (bottom right quadrant) and late phase (top right quadrant) was increased at the 40, 60, and 80 µg/mL of silymarin treatment. These observations indicated that the total number of apoptotic cells was 20.8% at the maximum dose of silymarin (80 µg/mL) which is markedly less than that induced by silymarin in the p53⁺/⁺ fibroblasts (61%). Thus, the analysis of apoptotic cells indicated that p53-deficient cells are less susceptible to silymarin-induced apoptosis, which support the notion that silymarin-induced apoptosis in preneoplastic epidermal JB6 C141 cells may be mediated primarily through the involvement of tumor suppressor protein p53 although p53-independent pathway may also have a role but seems less.

**Silymarin Down-Regulates Antiapoptotic Proteins Bcl-2 and Bcl-xl, but Up-Regulates Expression of Proapoptotic Protein Bax in JB6 C141 Cells**

The antiapoptotic proteins Bcl-2 and Bcl-xl have been associated with the inhibition of apoptosis and cell survival mechanisms. Bax protein is a proapoptotic member of this family and increased expression of this protein often associated with the increased apoptosis in target cells (26–28). We therefore reasoned that if p53 was involved in silymarin-induced apoptosis, there would be decreased expression of Bcl-2 and Bcl-xl and increased expression of Bax protein in silymarin-treated JB6 C141 cells. Treatment of JB6 C141 cells with silymarin resulted in a dose-dependent decrease in the levels of proapoptotic Bcl-2 and Bcl-xl proteins expression compared with nonsilymarin-treated cells. Silymarin treatment at the 60 and 80 µg/mL doses resulted in >90% inhibition of Bcl-2 (Fig. 6A, A) and Bcl-xl proteins (Fig. 6A, D). At the same time, the level of proapoptotic Bax protein was correspondingly up-regulated from 1.6- to 3.6-fold with increasing doses of silymarin under identical conditions (Fig. 6A, B). Because the ratio of Bax and Bcl-2 expression is the determining factor for the induction of apoptosis, we found that the ratio of Bax/Bcl-2 protein expression was significantly increased (P < 0.001) dose-dependently after silymarin treatment (C), which indicated the susceptibility of JB6 C141 cells towards apoptosis.

**Silymarin Induces Cytochrome c Release, Induction of Apaf-1, and Activation of Caspase-3 and PARP Cleavage in JB6 C141 Cells**

In biological system, apoptosis may involve disruption of mitochondrial function through the abnormal expression of Bcl-2 and/or Bax which induces the release of cytochrome c from mitochondria into the cytosol. Cytosolic cytochrome c can interact to Apaf-1 and leading to the activation of caspases in the apotosome and finally lead to the activation of caspase-3. Activation of caspase-3 subsequently leads to apoptosis (14, 15). For these reasons, we determined...
whether induction of apoptosis in JB6 C141 cells by silymarin is associated with disruption of mitochondrial function and activation of caspases. By Western blot analysis, we found that treatment of JB6 C141 cells with silymarin (20–80 μg/mL) resulted in a dose-dependent increase in cytochrome c release from mitochondria (Fig. 6B, A), induction of Apaf-1 (Fig. 6B, B), activation of caspase-3 (Fig. 6B, C) and PARP (Fig. 6B, D) proteins compared with nonsilymarin-treated control cells. Activated or cleaved caspase-3 (17 and 19 kDa) is crucial for the induction of apoptosis. Because cleaved caspase-3 antibody only recognizes the cleaved products of caspase-3 (17 and 19 kDa), the original basal level of uncleaved caspase-3 was not detected in the blot.

**Inhibition of Caspases Prevents Silymarin-Induced Apoptosis in JB6 C141 Cells**

Because we found that silymarin treatment increased the activation or cleavage of caspase-3, and this may involve in silymarin-induced apoptosis in JB6 C141 cells, we were interested to examine whether inhibition of
caspases will prevent silymarin-induced apoptosis in JB6 C141 cells. As shown in Fig. 6C, pretreatment of JB6 C141 cells with caspase inhibitor, Z-VAD-FMK (20 μmol/L; ref. 29) significantly inhibited silymarin-induced (40 and 60 μg/mL) apoptosis (>70%, P < 0.001) in these cells. This observation suggested that silymarin-induced apoptosis required activation of caspases, including caspase-3.

**Silymarin Decreases Bcl-2 Expression and Concomitantly Increases the Expression of Bax in p53+/+ Cells but not in p53−/− Cells**

Next, we were interested to determine the effect of silymarin on Bcl-2 and Bax protein expression in p53 wild-type and p53-deficient fibroblasts. Western blot analysis indicated that treatment of silymarin decreased the expression of Bcl-2 (4–60%) in the p53+/+ cells (Fig. 7, left A) but had little effect on Bcl-2 expression only at higher dose of silymarin (80 μg/mL) in the p53-deficient cells (Fig. 7, right D). Similarly, treatment of silymarin increased the expression of Bax protein in the p53 wild-type cells (Fig. 7, left B) but had no effects on its expression in p53-deficient cells (Fig. 7, right E). Again, the ratio of Bax/Bcl-2 proteins is a critical factor for apoptotic signal. Our data indicated that treatment of silymarin increased the ratio of Bax/Bcl-2 proteins by ~4-fold in p53+/+ fibroblasts compared with nonsilymarin-treated p53−/− fibroblasts (Fig. 7C). However, the ratio of Bax/Bcl-2 proteins was only about 1.5-fold in p53-deficient fibroblasts after only a higher dose of silymarin (80 μg/mL) treatment (Fig. 7F) compared with nonsilymarin-treated cells. This may be the reason that some cells were undergoing apoptosis in p53-deficient cells (Figs. 4 and 5). These observations provide evidence that p53 may have a role in silymarin-induced apoptosis.

**Discussion**

In earlier studies, we have shown that topical application of silymarin significantly inhibited chemical carcinogenesis and UV-induced skin carcinogenesis in terms of tumor incidence, tumor multiplicity, and tumor size (4). In our continuing efforts, we are trying to develop new and effective chemopreventive agents that can inhibit, reverse or slow down the risk of skin cancers. Chemoprevention is a promising strategy to control the occurrence of cancer. Several botanicals and/or dietary supplements have been investigated for their anti-skin carcinogenic properties. Silymarin, a plant flavonoid, is one of them and has been shown to have anticarcinogenic properties (2–5); however, the molecular mechanisms of the anti-skin carcinogenic effects of silymarin are still not clearly understood. In some in vitro studies, it has been shown that there is not significant difference in between the anticarcinogenic effect of silymarin and silibinin (30). In our preliminary studies conducted with JB6 C141, p53+/+, and p53−/− cells, we found that treatment of silymarin and silibinin at the equal doses induced almost similar inhibitory effect on cell viability (Fig. 1 and Results section). Furthermore, the cost of silibinin is thrice greater than silymarin as can be seen in catalog of Sigma Chemical Co. Silibinin is not easily soluble in water or organic solvents like acetone etc. but soluble in DMSO. DMSO is not considered as an appropriate solvent for skin care lotions or creams. We also believe that the effect of silymarin could be better in comparison to silibinin because of synergistic effect of all the constituents present in silymarin compared with silibinin alone. Because of this reasoning, we used silymarin in our present study. Treatment of silymarin to preneoplastic JB6 C141 cells induced apoptosis (Fig. 2A and B). Inhibition of apoptosis is considered as one of the possible mechanisms of tumor development and many chemopreventive agents have been shown to act through the induction of apoptosis to inhibit the carcinogenic process (31, 32). Therefore, the induction of apoptosis in JB6 C141 cells may be one of the mechanisms of the anticarcinogenic effect of silymarin. It has been suggested that apoptosis may represent a protective mechanism against neoplastic development by eliminating genetically damaged cells or excess cells that have improperly been induced to proliferate (33, 34).

The apoptosis has been shown to be regulated by tumor suppressing activity of p53 protein (35, 36). Studies have indicated that the lack of p53 expression or function is associated with an increased risk of tumor formation (37, 38). p53 phosphorylation has been associated with cellular damage occurring during anticancer therapy (39). To investigate the possible role of p53 in silymarin-induced apoptosis, we used preneoplastic epidermal

**Figure 7.** Treatment of silymarin decreases the expression of antiapoptotic protein Bcl-2 and increases the expression of proapoptotic protein Bax in p53−/− fibroblasts (A and B). In p53-deficient cells however, the levels of Bcl-2 (D) decreased only at the maximum concentration of silymarin treatment whereas the level of Bax (E) remained almost unchanged. Representative blot from three independent experiments with identical results. Numbers below the bands, relative intensity of bands in that blot. Columns, mean ratios of Bax and Bcl-2 protein expression in p53−/− and p53+/+ fibroblasts were produced from three separate experiments; bars, ±SD (C and F). Cells were cultured and treated with silymarin as described in Fig. 6, and the protein levels were analyzed by Western blot analysis as described in Materials and Methods. *, P < 0.05 versus control.
keratinocytes JB6 C141 cells, and p53+/+ and p53−/− fibroblast cell lines. It is evident from our data that the basal level of p53 and the level of phosphorylation of p53 at Ser15 were increased after silymarin treatment which suggested that the induction of apoptosis by silymarin is mediated via p53 protein expression (Fig. 3). It has also been shown that phosphorylation of p53 at Ser15 increases its half-life and thus increases its accumulation and functional activation of p53 in response to DNA damage (40, 41). We were further interested to confirm whether p53 is involved in silymarin-induced apoptosis. For this purpose, we compared the effect of silymarin in wild-type p53 fibroblasts and p53-deficient fibroblasts. Our data indicates that treatment of p53+/+ cells with silymarin resulted in significant induction of apoptosis (Figs. 4 and 5) whereas significantly less number of apoptotic cells was observed in p53−/− cells (Figs. 4 and 5). These results provide supporting evidence for the requirement of p53 in silymarin-induced apoptosis. Venkatachalam et al. (42) have shown that loss of tumor suppressor gene function is associated with amelioration of apoptosis and increased growth of tumors. Moreover, it has also been shown that mouse fibroblasts deficient for p53 were more sensitive to induction of apoptosis due to the direct involvement of p53 in DNA repair and suggested that apoptotic pathway may be dependent on the cell type (43). Our observations as well as the evidence from others, however, indicate the probability of the induction of apoptosis via the p53 pathway is dependent both on the cell type and on the ability of the chemopreventive agent being tested to induce apoptosis (19, 20). Several pathways mediate p53-induced apoptosis, and one of these involves the Bcl-2 and Bax proteins. Bax protein is a p53 target and a proapoptotic member of the Bcl-2 family (44, 45). The Bcl-2 family consists of both proapoptotic and antiapoptotic members that elicit opposing effects on mitochondria. Bax can promote the release of cytochrome c into the cytosol from mitochondria, which in turn activates caspase-3, one of the key executioners of apoptosis and PARP (16). The antiapoptotic proteins such as Bcl-2 and Bcl-xl, which are transcriptionally suppressed by p53, preserve the integrity of the mitochondria (46). This blocks the release of cytochrome c that activates the effectors of apoptosis (47). We observed that silymarin up-regulates the expression of proapoptotic protein Bax and down-regulates the expression of antiapoptotic protein Bcl-2 in JB6 C141 cells (Fig. 6A) and in p53+/+ fibroblasts (Fig. 7), but this effect was not observed in p53-deficient cells (p53−/−; Fig. 7). Importantly, the ratio of pro- and antiapoptotic protein expression, such as Bax/Bcl-2, is critical for the induction of apoptosis, and the ratio of Bax/Bcl-2 decides a cell’s susceptibility to undergo apoptosis (16). Change in the ratio of Bax/Bcl-2 stimulates the release of cytochrome c from mitochondria into cytosol. Cytosolic cytochrome c can interact with Apaf-1 and leads to the activation of caspase-3 and PARP (14, 15). In this study, we found that treatment of silymarin results in an increase in Bax/Bcl-2 ratio in JB6 C141 cells (Fig. 6A), increased release of cytochrome c, increased expression of Apaf-1, and finally increased expression of cleaved caspase-3 (Fig. 6B). Cleaved caspase-3 results in increased apoptosis in JB6 C141 cells. The role of caspase activation in silymarin-induced apoptosis was further confirmed when we observed that treatment of caspase inhibitor, Z-VAD-FMK, prevented silymarin-induced apoptosis in JB6 C141 cells (Fig. 6C). Similar to JB6 C141 cells, treatment of silymarin to p53+/+ fibroblasts also results in about 4-fold increase in Bax/Bcl-2 protein ratio, which may be responsible for the induction of apoptosis. The modest increase in Bax/Bcl-2 ratio was also observed in p53−/− fibroblasts but only at the higher dose of silymarin (80 μg/mL; Fig 7F), and this may be the reason for small degree of apoptosis observed in p53−/− cells. These results provide evidence for the importance of p53 and its phosphorylation at Ser15 in silymarin-induced apoptosis.

In conclusion, the results of the present study indicate that silymarin-induced apoptosis in JB6 C141 cells primarily mediated through a p53-dependent pathway, and p53-independent pathway seems to have a minor role. p53-dependent pathway involves the proteins of Bcl-2 family, cytochrome c, Apaf-1, and activation of caspase-3 and PARP. Further studies are in progress to define the role of p53-independent pathway in induction of silymarin-induced apoptosis in JB6 C141 cells. However, we suggest that induction of p53-mediated apoptosis is, at least in part, a possible explanation for the anti-skin carcinogenic effect of silymarin, and that silymarin has a potential to prevent cancer.

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References

Silymarin Induces Apoptosis via p53-Dependent Pathway

Silymarin induces apoptosis primarily through a p53-dependent pathway involving Bcl-2/Bax, cytochrome c release, and caspase activation

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