Silymarin inhibits growth and causes regression of established skin tumors in SENCAR mice via modulation of mitogen-activated protein kinases and induction of apoptosis

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This study reports in vivo therapeutic efficacy of silymarin against skin tumors with mechanistic rationale. 7,12-Dimethylbenz[a]anthracene–12-O-tetradecanoyl-phorbol-13-acetate (DMBA–TPA)-induced established skin papilloma (tumor)-bearing SENCAR mice were fed with 0.5% silymarin in AIN-93M-purified diet (w/w), and both tumor growth and regression were monitored during 5 weeks of feeding regimen. Silymarin feeding significantly inhibited (74%, P < 0.01) tumor growth and also caused regression (43%, P < 0.01) of established tumors. Proliferating cell nuclear antigen and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling immunohistochemical staining of tumors showed that silymarin decreases proliferation index by 48% (P < 0.001) and increases apoptotic index by 2.5-fold (P < 0.001), respectively. Skin tumor growth inhibition and regression by silymarin were also accompanied by a strong decrease (P < 0.001) in phospho-ERK1/2 levels in tumors from silymarin-fed mice compared with controls. In the studies evaluating bioavailability and physiologically achievable level of silymarin (as silibinin) in plasma, skin tumor, skin, liver, lung, mammary gland and spleen, we found 10, 6.5, 3.1, 13.7, 7.7, 5.9 and 4.4 µg silibinin/ml plasma or per gram tissue, respectively. In an attempt to translate these findings to human skin cancer and to establish biological significance of physiologically achievable level, effect of plasma concentration of silibinin was next examined in human epidermoid carcinoma A431 cells. Silibinin treatment of cells in culture at 12.5, 25 (plasma level) and 50 µM doses resulted in 30–74% (P < 0.01–0.001) growth inhibition and 7–42% death of A431 cells in a dose- and time-dependent manner; apoptosis was identified as a cell death response by silibinin. Similar silibinin treatments also resulted in a significant decrease in phospho-mitogen-activated protein kinase/extracellular signal-regulated protein kinase 1/2 (MAPK/ERK1/2) levels, but an up-regulation of stress-activated protein kinase/jun NH2-terminal kinase (SAPK/JNK1/2) and p38 mitogen-activated protein kinase (p38 MAPK) activation in A431 cells. The use of MEK1 inhibitor, PD98059, showed that inhibition of ERK1/2 signaling, in part, contributes to silibinin-caused cell growth inhibition. Together, the data suggest that an inhibition of ERK1/2 activation and an increased activation of JNK1/2 and p38 by silibinin could be possible underlying molecular events involved in inhibition of proliferation and induction of apoptosis in A431 cells. These data suggest that silymarin and/or its major active constituent silibinin could be an effective agent for both prevention and intervention of human skin cancer.

Introduction

The role of dietary modification in the reduction of cancer risk has recently drawn widespread attention, because the differences in worldwide human cancer mortality often depend on lifestyle and dietary habits (1–3). As a result of the unavoidable presence of mutagens and carcinogens in our environment and also in the human diet, it has been suggested that dietary intake of phytochemicals, including antioxidants, could be a useful strategy against the deleterious effects of these mutagenic and carcinogenic agents (4). Diets rich in naturally occurring polyphenolic flavonoids have been associated with the reduced incidence of certain human cancers (1–10). Among these, silymarin, isolated from the fruits of milk thistle, Silybum marianum L. Gaertn. (11), is being used clinically as an anti-hepatoxic agent for the treatment of various liver diseases in Europe and Asia (9,11) and recently, has also been marketed in the USA and Europe as a dietary supplement. Silymarin is composed mainly of silibinin with small amounts of other silybinin stereoisomers, namely, isosilybin, dihydrosilybin, silydianin and silychristin (12). Recent studies with silymarin showed that it could be used against a wide range of liver and gall bladder diseases, including hepatitis and cirrhosis as well as dermatological conditions (13–15). Studies on various animal models using different modes of administration of silymarin showed that it is non-toxic and largely free of adverse side effects in subchronic and chronic tests even at large doses, and also there is no known LD50 in laboratory animals (16). Several studies in rodents and cell cultures have shown that silymarin is a strong antioxidant and scavenges both free radicals and reactive oxygen species, and thereby provides significant protection against different cancers of epithelial origin (17–19). It has also been shown that silymarin inhibits the 12-O-tetradecanoyl-phorbol-13-acetate (TPA)-induced anchorage-independent growth of JB6 mouse epidermal cells (20), 7,12-dimethylbenz[a]anthracene (DMBA)–TPA-induced mammary lesion formation in organ culture (21) and formation of transformed rat tracheal epithelial cell colonies induced by exposure to benzo[a]pyrene (22).

We have recently demonstrated the strong cancer chemo-
preventive effects of silymarin using different long-term skin tumorigenesis animal protocols as well as short-term cell culture systems (5,23–26). Studies in our laboratory have shown that silymarin strongly inhibits ornithine decarboxylase activity and mRNA expression as well as TNF-α mRNA expression induced by structurally different tumor promoters, including free radical-generating compounds, in mouse epidermis (24,26). In another study we have shown that silymarin affords strong protection against UV-B radiation-induced tumor initiation, tumor promotion and complete carcinogenesis in SKH-1 hairless mouse skin (23). A mechanistic study has shown that silymarin inhibits TPA-caused induction of cyclooxygenase (COX) 2 and interleukin-1α expression in SENCAR mouse epidermis (25). Recently, we showed that the skin cancer preventive effect of silymarin involves the impairment of epidermal growth factor receptor (EGFR)-mediated signaling (27,28).

After establishing that silymarin is strongly effective in inhibiting the initial stages of skin tumor development, the aim of the present study was to ascertain its anticancer effect on DMBA–TPA-induced established skin papillomas (tumors) in SENCAR mice, and possible implications of such findings for human skin cancer intervention at actinic keratosis stage, a benign skin cancer condition. First, we studied in vivo therapeutic effect of silymarin in DMBA–TPA-induced established mouse skin tumors and associated molecular mechanisms. Next, we examined whether the mechanism-based anticancer effect of silymarin (as its major active constituent silibinin) is operative in human epidermoid carcinoma A431 cells. We also examined the levels of dietary administered silymarin (as silibinin) in plasma, skin tumors, skin and some other organs which are susceptible to the development of cancer to evaluate its bio-distribution, and to determine pharmacologically and physiologically effective doses for further advanced studies. Here, we present the evidence that silymarin effectively suppresses tumor growth and causes regression of established skin tumors in vivo, possibly via inhibition of mitogen-activated protein kinase/extracellular signal-regulated protein kinase 1/2 (MAPK/ERK1/2) activation leading to a decrease in proliferation and an induction of apoptosis in tumor cells. Furthermore, it shows a strong anticancer effect against A431 cells via similar mechanisms together with an activation of stress-activated protein kinase/jun NH2-terminal kinase (SAPK/JNK1/2) and p38 mitogen-activated protein kinase (p38 MAPK), making it an excellent candidate for an anticancer agent against human skin cancer.

Materials and methods

Animals, diets and chemicals
Six-week-old female SENCAR mice were obtained from the National Cancer Institute (Frederick Cancer Research and Development Center, Frederick, MD) and housed at standard laboratory conditions (temperature 24 ± 2°C, relative humidity 50 ± 10% and 12 h room light/12 h dark cycle). They were acclimatized for 1 week before starting the present study, and fed with Purina Chow diet and water ad libitum. DMBA was from Aldrich Chemical Co. (Milwaukee, WI) and silymarin, silibinin and TPA were from Sigma Chemical Co. (St Louis, MO). The silymarin dose used in this study was 0.5% (w/w) in AIN-93M-purified rodent diet (pelleted) prepared by Dyets Inc. (Bethlehem, PA). All other chemicals were obtained in the purest form available commercially.

Cell line and reagents
Human epidermal carcinoma cell line A431 was from the American Type Culture Collection (Manassas, VA). Cells were grown in Dulbecco’s modified Eagle medium (DMEM; Gibco BRL, Grand Island, NY) with 10% fetal bovine serum (HyClone, Logan, UT) and 1% penicillin–streptomycin (Gibco BRL) at standard culture conditions. Phospho- (and regular) MAPK/ERK1/2 (Thr202/Tyr204) antibodies were from New England Biolabs (Beverly, MA). Phospho-p38 MAPK (Thr180/Tyr185) and p38 MAPK (Thr180/Tyr182) antibodies were purchased from Cell Signaling Technology (Fremont, CA). Anti-rabbit immunoglobulin horseradish peroxidase-conjugated secondary antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). PD98059 was from Alexis Biochemicals (San Diego, CA). ECL detection system was from Amersham (Arlington Heights, IL).

Formation of established skin papillomas in SENCAR mice and treatment with silymarin
For the formation of established skin papillomas in SENCAR mice, a recently published protocol by Lu et al. was used (29). Briefly, SENCAR mice were treated with a single topical application of DMBA (200 nmol) in 200 µl acetone on to the shaved dorsal skin of each mouse. After 1 week, tumor initiated skin was promoted with 5 nmol TPA (in 200 µl acetone) per mouse twice a week for 24 weeks, at which point 100% of mice showed established skin papillomas (29), hereafter referred to as skin tumors. To determine the effect of silymarin on established skin tumor growth at 24 weeks, mice were transferred from a regular chow diet to a more defined AIN-93M-purified diet for 5 days before starting silymarin treatment. Mice having six to eight tumors were randomly divided into two groups of nine to 10 animals each and one group of animals was continued on the AIN-93M diet and served as control whereas the other group of animals was fed with the AIN-93M diet containing 0.5% silymarin (w/w) for 5 weeks. Body weight was recorded throughout the experiment, and tumor sizes were measured twice weekly and tumor volume calculated by: \(4\pi r^3/3\), where \(r^2\) is the mean radius of two dimensions of each tumor. With regard to tumor study, data from the control or silymarin-fed group of mice were analyzed for: (i) tumor volume/mouse that represents total tumor burden in terms of volume/mouse; (ii) tumor volume/tumor that represents the average volume of each tumor from all the tumors in each mouse; and (iii) a comparison between overall change in tumor volume/mouse at the start (0 week) and end (5 weeks) of dietary feeding.

At the end of the experiment, blood was collected intracardially for high performance liquid chromatography (HPLC) analysis of silymarin level in plasma samples. Additionally, tumors, skin, liver, lung, mammary gland and spleen were also collected to estimate bio-distribution of dietary fed silymarin. All tumors were stored as desired and processed for molecular and immunohistochemical studies as needed.

Immunohistochemical detection of proliferating cell nuclear antigen in skin tumors
Briefly, tumor samples were fixed in 10% buffered formalin for 24 h and processed conventionally. Paraffin-embedded tumor sections (5 µm thick) were heat immobilized, and deparaffinized using xylene and rehydrated in a graded series of ethanol with a final wash in distilled water. Antigen retrieval was carried out in 10 mM citrate buffer (pH 6.0) in a microwave for 2 and 1.5 min at full and 20% of power levels, respectively. Endogenous peroxidase activity was blocked by immersing the sections in 3% H2O2 in methanol (v/v) followed by three changes in 10 mM phosphate-buffered saline (PBS, pH 7.4). Sections were then incubated with mouse monoclonal anti-proliferating cell nuclear antigen (PCNA) antibody IgG2a (Dako, Carpinteria, CA), 1:400 in PBS for 1 h at 37°C in humidity chamber. Negative controls were treated only with PBS under identical conditions. Sections were then incubated with biotinylated rabbit anti-mouse antibody IgG (1:200 in 10% normal rabbit serum from Dako) for 30 min at room temperature. Thereafter, following wash with PBS, sections were incubated with conjugated horseradish peroxidase streptavidin (Dako), 1:1000 in PBS for 30 min at room temperature in a humidity chamber. Sections were then incubated with 3,3′-diaminobenzidine (DAB; Sigma) working solution for 10 min at room temperature and counterstained with diluted Harris hematoxylin for 2 min, and rinsed in Scott’s water.

Slides were then dehydrated, mounted, viewed and photographed using Zeiss light microscope (Germany). Proliferating cells were quantified by counting PCNA-positive cells and total number of cells at 10 arbitrarily selected fields at 400-fold magnification in a double-blinded manner. The proliferation index (per 400-fold microscope field) was determined as number of PCNA-positive cells×100/total number of cells.

In situ apoptosis detection in skin tumors by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling
First, formalin-fixed and paraffin-embedded 5 µm thick sections of tumor samples (those used for PCNA staining) were used for conventional H&E staining to observe the cellular morphological changes. Next, in order to identify early as well as late apoptotic cells, tumor samples were also studied by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.
Silymarin inhibits growth of established skin tumors

(TUNEL) staining. DNA fragmentation in the individual apoptotic cell was visualized by detection of biotinylated nucleotides incorporated into the free 3'-hydroxyl residues of these DNA fragments by Tumor TACS In Situ Apoptosis Detection Kit (R & D Systems, Minneapolis, MN). Briefly, tumor samples were first fixed with 10% buffered formalin for 8 h to prevent the loss of low molecular weight DNA fragments; then paraffin-embedded 5 µm thick sections were cleared in xylene and rehydrated in graded concentrations of ethanol. Slides were rinsed with Ca²⁺, Mg²⁺ and DNAAse-free PBS (10 mM PBS, pH 7.4) and permeabilized with proteinase K at room temperature to make DNA accessible to labeling enzyme. For positive control, sections were incubated with 10 µg/ml of each of Caspase-3 and -7, which generates breaks in virtually every cell. Endogenous peroxidase activity was quenched using 5% H₂O₂ in (methanol, v/v) for 5 min and sections were incubated with TdT (terminal deoxynucleotidyl transferase) labeling buffer for 5 min before starting the labeling reaction. Sections were then incubated with TdT enzyme and biotinylated nucleotides (for negative control, labeling buffer was used instead of TdT enzyme) for 1 h at 37°C in humidified chamber. The reaction was stopped by adding TdT stop buffer for 5 min. Sections were incubated with streptavidin-conjugated horseradish peroxidase for 10 min. Brown color was developed by incubation in DAB working solution (1:5:5000; 30% H₂O₂: DAB:1×PBS) for 7 min at room temperature. Slides were counterstained in 1% methyl green for 1 min and visualized and scored under a light microscope. Apoptosis was evaluated by counting positive cells (brown staining) and the number of cells at arbitrarily selected fields at 400-fold magnification in a double-blinded manner. The apoptotic index (per 400-fold microscope field) was calculated as number of apoptotic cells × 100/total number of cells.

MAPK/ERK1/2 study in skin tumors

Briefly, one piece of tumor, collected from each mouse at the termination of the experiment, was frozen in liquid nitrogen and powdered by mortar and pestle. Tumor samples (100 mg each) were added with 200 µl of lysis buffer (10 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 0.5 mM phenyl methyl sulfonyl fluoride, 0.2 mM sodium orthovanadate, 0.5% NP-40, 5 U/ml aprotinin), homogenized at 4°C and kept on ice for 30 min followed by centrifugation at 15 000 r.p.m. for 30 min. Protein concentration was determined in clear supernatants (tumor lysates) using Bio-Rad DC (Hercules, CA) protein assay kit. For western immunoblotting, 70 µg protein/tumor lysate from both control- and silymarin-fed groups were denatured with 2% sample buffer and subjected to SDS–PAGE on a 12% gel. Separated proteins were transferred on to nitrocellulose membrane, and probed with anti-phospho-MAPK/ERK1/2 and anti-MAPK/ERK1/2 antibodies followed by peroxidase-conjugated appropriate secondary antibody and visualization by ECL detection system.

HPLC analysis for bio-distribution of dietary administered silymarin (as silibinin) in different organs and plasma of mice

At the end of 5 weeks of dietary feeding of silymarin to the mice with established skin tumors, all the mice were killed and the blood was collected intracardially to sample plasma samples. Additionally, to assess the bio-distribution of silymarin, different organs (skin tumor, skin, liver, lung, mammary gland and spleen) were also collected and frozen in liquid nitrogen and stored at –80°C until further processing. For the extraction of total silibinin (free and conjugated forms), 100–200 mg of tissue samples were thoroughly homogenized in 3 vol of 50 mM Tris–HCl (pH 7.4) at 4°C using an Polytron PT-10 homogenizer (VWR Scientific, Painfield, NJ). Thereafter, each tissue homogenate or plasma sample (100 µl) was mixed with 10 µl of an ascorbate–EDTA solution (20% ascorbic acid and 0.01% EDTA dissolved in a 0.4 M sodium phosphate buffer, final pH 3.6), and a mixture of β-glucuronidase (250 U) and sulfatase (20 U). The reaction mixture was then incubated at 37°C for 45 min and extracted twice with ethyl acetate. The ethyl acetate extracts were pooled and evaporated to dryness in a vacuum centrifuge concentrator. The residues obtained were re-dissolved in 20 µl of 10% acetonitrile aqueous solution and subjected to centrifugation. The resultant clear supernatants were stored at –80°C until HPLC analysis.

Standard silibinin and the ethyl acetate extracts of plasma and tissue samples were analyzed on HPLC system (ESA, Bedford, MA) using C18 reversed phase analytical column (3 µm, 4.6×250 mm). HPLC mobile phase used was solvent A (0.05% v/v acetic acid in distilled water) and solvent B (100% acetonitrile). The linear gradient system, employed at room temperature, was: 0–5 min, 100% solvent A; 5–10 min, 100% solvent A to 70% solvent A and 30% solvent B; 10–30 min, 70% solvent A and 30% solvent B to 30% solvent A and 70% solvent B; 32 min, stop of run. The solvent flow rate throughout the run was 1 ml/min. The column eluate was monitored by UV absorbance at 270 nm. The silibinin peak in plasma, and other tissue samples, was identified by comparing their HPLC retention time with authentic standard silibinin under identical conditions.

Before the analysis and quantification of silibinin extracted from plasma and tissue samples, different concentrations of standard silibinin were analyzed by HPLC to find a quantitative linear range for UV detection. Silibinin in samples was quantified on the basis of peak area under the curve calculations and comparison with standard silibinin. The recovery of silibinin after extraction from plasma and tissue homogenates was checked by adding a known amount of silybin to plasma and each tissue homogenate, and following vigorous mixing, its extraction and quantification by HPLC as detailed above.

Cell growth and viability assays

Human epidermoid carcinoma A431 cells were plated at 5000 cells/cm² in 35 mm dishes under the culture conditions described above. At 25–30% confluency, cells were fed with fresh medium and treated with either DMSO alone (control) or different concentrations (12.5, 25 and 50 µM) of silybin in DMSO. At 24, 48 and 72 h after the treatment, both floating and attached cells were collected by trypsinization, and counted in duplicate with a hemocytometer. Trypan blue dye exclusion was used to determine the cell viability. Each treatment and time point had two plates. The representative data shown in two independent experiments. Silybin treatment of A431 cells, and MAPK/ERK1/2, SAPK/JNK1/2 and p38 MAPK studies

A431 cells were grown in 100 mm dishes, as described above, and at 40–50% confluency were treated with either DMSO alone or different concentrations (12.5, 25 and 50 µM) of silybin in DMSO. After 24 and 48 h of these treatments, medium was aspirated and cells were washed twice with ice-cold PBS. Two hundred microliters of lysis buffer (used in skin tumor study) was added in each dish and kept at 4°C for 15 min. Cell lysates were scraped and kept on ice for an additional 20 min followed by clearing of lysate and protein estimation as described above. For western immunoblotting, 50 µg protein/sample lysate was used as described in the skin tumor study.

FACS analysis for apoptotic death of A431 cells

Briefly, at ~30% confluency (in culture conditions described above) cells were treated with either DMSO alone (control) or varying concentrations (12.5, 25 and 50 µM) of silybin. After 48 and 72 h of these treatments, both floating and attached cells were collected, and a quantitative apoptotic death assay was performed by annexin V and propidium iodide (PI) staining followed by FACS analysis using Vybrant Apoptosis Assay Kit 2 (Molecular Probes, Eugene, OR) and vendor’s protocol as described recently (30). The kit contains recombinant annexin V conjugated to fluorophores and Alexa Fluro 488 dye having almost perfect spectral match to fluorescein and comparatively greater sensitivity. In apoptotic cells, annexin V binds to phosphatidylserine, which is translocated from inner to outer leaflet of the plasma membrane.

3-[4,5-Diaminohexyl]-2-fluorophenyltetrazolium bromide assay

Briefly, 5000 A431 cells/well were plated in 96 well plates under the culture conditions described above, and treated either with vehicle (0.1% DMSO v/v), or with silybin (50 µM), or MEK1 inhibitor PD98059 (50 µM) or both for 24 h. At the end of the treatment, cells were washed twice with 1× PBS and incubated with 1 mg 3-[4,5-diaminohexyl-2-fluoro]-2,5-diphenyl-tetrazolium bromide (MTT)/ml of serum-free media for 2 h. Finally, treatment medium was aspirated out and DMSO was added in each plate, and absorbance of the colored color was taken at 590 nm against DMSO using a 96 well-microplate reader. Similar silybin and PD98059 doses were also used for cell growth assay as described above.

Densitometric analysis and statistical evaluation

 Autoradiograms of western immunoblots were scanned with Adobe Photoshop (Adobe Systems, San Jose, CA), and adjusted for brightness and contrast for minimum background. Mean density of each band was analyzed by Scanimage Program (NIH, Bethesda, MD). In each case, densitometric analysis data for phospho-ERK1/2, JNK1/2 and p38 blots were corrected for loading with the density of total ERK1/2, JNK1/2 and p38 blots respectively. For statistical evaluation, data were analyzed using Jandel Scientific SigmaStat 2.0 software package. For all measurements, Student’s two-tailed t-test was employed to assess statistical significance of difference between control and silymarin (or silibinin)-treated groups. A statistically significant difference was considered to be present at P < 0.05.

Results

Growth inhibition and regression of established skin tumors in SENCAR mice by silymarin

The important observation was that dietary feeding of silymarin at 0.5% (w/w) dose to mice bearing established skin tumors did not show any apparent sign of toxicity as monitored by
body weight gain profile (Figure 1A). The growth of established mouse skin tumors, maintained for 24 weeks through the DMBA–TPA initiation–promotion protocol, was significantly inhibited by silymarin feeding (0.5%) in a purified AIN-93M diet. The mean tumor volume per mouse showed a 6, 18, 32, 68 and 74% (P < 0.05–0.01) decrease at 1–5 weeks of silymarin feeding, respectively, as compared with the control group of animals (Figure 1B). In the same treatment regimen, mean tumor volume per tumor was reduced by 12, 28, 50, 78 and 83% (P < 0.05–0.01) (Figure 1C). The strong growth inhibitory effect of silymarin on established skin tumors was also accompanied by the regression of these tumors. At the end of 5 weeks of silymarin feeding, tumor volume per mouse and tumor volume per tumor were reduced by 43 and 39% (P < 0.05), respectively, as compared with start of silymarin treatment (Figure 1D).

Therapeutic effect of silymarin against established mouse skin tumors is associated with its in vivo anti-proliferative and apoptotic efficacy

In order to assess the in vivo effect of silymarin feeding to mice and its anti-proliferative and apoptotic responses towards observed inhibition of tumor growth accompanied with regression of established skin tumors, tumor samples were analyzed for PCNA, H&E and TUNEL staining. The qualitative immunohistochemical localization of PCNA in proliferating cells is shown by microscopic images of different tumor sections stained with anti-PCNA antibody (Figure 2A and B). As observed by visual examination of the PCNA-stained cells, compared with AIN-93M diet-fed controls (Figure 2A), a strong reduction in PCNA-stained cells was clearly evident in tumor samples from mice fed with silymarin (Figure 2B). Tissue sections where PBS was used instead of the PCNA monoclonal antibody (negative controls) did not show noticeable positive staining (data not shown). As shown by quantitative analysis of PCNA immunohistochemical staining data, silymarin feeding to mice bearing established skin tumors resulted in 48% (P < 0.001) decrease in proliferation index as compared with the control group of skin tumors (Figure 2C).

H&E staining of tumor sections showed the presence of apoptotic bodies without any significant alteration in the cellular morphology of tumor cells following silymarin feeding (data not shown). Based on H&E staining results that subjectively suggested the possibility of an apoptotic response by silymarin in tumors, TUNEL staining was next performed to further substantiate in vivo apoptotic efficacy of silymarin in these tumors. Qualitative TUNEL staining in apoptotic cells is shown by microscopic images of different tumor tissue sections stained with TdT enzyme (Figure 2D and E). As observed by visual examination of TUNEL-stained cells, compared with controls (Figure 2D), a strong increase in TUNEL-stained cells was clearly evident in tumor samples following silymarin feeding (Figure 2E). The positive control samples, in which TACS-nuclease was used to generate DNA fragments with free 3'-OH ends, showed positive staining in all nuclei whereas the negative control, in which labeling buffer was used instead of the TdT enzyme, did not show any significant positive staining (data not shown). As shown by quantitative analysis of TUNEL-stained cells, silymarin strongly increased the apoptotic index in tumors accounting for a 2.5-fold (P < 0.001) increase as compared with that of the control group of tumors (Figure 2F).

Fig. 1. Therapeutic effect of silymarin against established skin tumors in SENCAR mice without any apparent sign of toxicity. As detailed in the ‘Materials and methods’, established skin papillomas (skin tumors) were induced by DMBA–TPA and maintained for 24 weeks. Mice bearing established skin tumors were fed with 0.5% silymarin in purified AIN-93M diet for 5 weeks. Body weight and tumor sizes were measured twice weekly. Body wt/mouse (A), tumor volume/mouse (B) and tumor volume/tumor (C) were plotted as a function of number of weeks of silymarin treatment. Tumor regression was shown in terms of tumor volume at the start of dietary feeding, and at the end of 5 weeks of control and silymarin diet (D). All data shown are mean ± SE of nine to ten mice in each group.
Silymarin inhibits growth of established skin tumors

Fig. 2. Therapeutic effect of silymarin against established skin tumors is associated with its \textit{in vivo} anti-proliferative and apoptotic efficacy. At the end of the experiment, detailed in Figure 1, tumor tissues were analyzed with anti-PCNA antibody and TUNEL staining for both qualitative and quantitative analysis of PCNA-positive and apoptotic cells, respectively, as detailed in ‘Materials and methods’. The proliferative and apoptotic indices were calculated by counting the PCNA- and TUNEL-positive cells in each section at 10 arbitrarily selected fields at 400× magnification as detailed in ‘Materials and methods’. The data shown are: the pictomicrographs to visually demonstrate the anti-PCNA antibody staining in control (A) and silymarin-treated tumors (B) and summary of quantitative analysis of proliferation index (C). Similarly, TUNEL staining data shown are: the pictomicrographs to visually demonstrate the TUNEL staining in control (D) and silymarin-treated tumors (E) and summary of quantitative analysis of apoptotic index (F). (A, B, D and E) Representative data are shown at 400× magnification. The quantitative proliferation and apoptotic indices data shown are mean ± SD of six to seven tumor samples from an individual mouse in each group.

Anti-proliferative and apoptotic effects of silymarin are accompanied by an inhibition of MAPK/ERK1/2 activation in skin tumors

As silymarin feeding to mice led to a decrease in tumor burden and regression of established skin tumors by inhibiting tumor cell proliferation and causing their apoptotic death, we next examined whether these effects in tumors are associated with an \textit{in vivo} inhibition of mitogenic signaling. For this, we assessed the levels of both phosphorylated and total MAPK/ERK1/2 in the tumors collected from both control and silymarin-fed mice at the termination of the experiment. Silymarin showed a profound \textit{in vivo} inhibitory effect on MAPK/ERK1/2 activation as evidenced by a strong decrease in phospho-ERK1/2 levels (Figure 3A, upper panel). As shown in Figure 3B, silymarin feeding for 5 weeks resulted in a 72 and 52% ($P < 0.001$) decrease in the phosphorylated levels
Fig. 3. Inhibitory effect of silymarin on the activation of MAPK/ERK1/2 in skin tumor of SENCAR mice. At the end of the experiment, detailed in Figure 1, tumor tissues were homogenized and cell lysates prepared as detailed in the ‘Materials and methods’. Cell lysates were subjected to SDS-PAGE followed by western blotting, as described in the ‘Materials and methods’. The membranes were probed with anti-phospho-ERK1/2 and regular ERK1/2 antibodies and then peroxidase-conjugated appropriate secondary antibody. Proteins were visualized with ECL detection system. (A) Phosphorylation of ERK1/2 (upper panel), total levels of ERK1/2 (bottom panel). Treatments are as labeled in the figure. Densitometric analysis for phospho-ERK1 and phospho-ERK2 (B) blots was corrected for loading with the density of ERK1/2 blots. The densitometric data shown are mean ± SD of four tumor samples from an individual mouse in each group which is representative of five to seven tumor samples from an individual mouse in each group.

of ERK1/2 as compared with the control group of tumors, respectively. This highly significant inhibition of ERK1/2 activation was not due to a change in total ERK1/2 protein level (Figure 3A, bottom panel).

Therapeutic effect of silymarin against established skin tumors and its in vivo anti-proliferative and apoptotic efficacy are at physiologically achievable doses

After establishing the therapeutic effect of silymarin against established skin tumors in SENCAR mice and its in vivo association with anti-proliferative and apoptotic efficacy, we next asked the question: what is the physiological dose of silymarin in animal study and whether this dose could be effective against human epidermoid carcinoma cells? To determine physiological concentration of silymarin following its addition to the diet, we focused on the level of silibinin that is the major biologically active constituent present in silymarin (12). First, a HPLC profile of silibinin was developed as a standard and its retention time was determined to be ~10.8 min (Figure 4A). Based on this HPLC profile of silibinin, a linear detection range of silibinin was also established (data not shown). The silibinin concentrations in plasma, skin tumor, skin, liver, lung, mammary gland and spleen were then calculated under linear range of detection employing area under curve of silibinin peak in HPLC profiles of these samples following enzyme digestion and ethyl acetate extraction (Figure 4B–H). As shown in Figure 4I, compared with control samples showing undetectable levels of silibinin (data not shown), silymarin feeding resulted in 10, 6.5, 3.1, 13.7, 7.7, 5.9 and...
Physiological level of silibinin induces apoptotic death in A431 cells

Based on the results showing that silibinin induces significant death of A431 cells, next we studied whether this is an apoptotic death? A quantitative apoptotic cell death study was performed to answer this question. Silibinin treatment at 12.5, 25 and 50 µM doses for 48 and 72 h resulted in time- and dose-dependent increase in apoptotic death of A431 cells. The number of early apoptotic (annexin V-stained) cells increased from 2.9% (control) to 3.4, 5.8 and 12.8% following silibinin treatment for 48 h at 12.5, 25 and 50 µM doses, respectively (Figure 6A). Similar silibinin treatment for 72 h increased the early apoptotic cells from 3.1% (control) to 3.7, 4.4 and 17.4%, respectively (Figure 6A). Membrane blebbing was also observed in annexin V-stained cells as an early apoptotic response by silibinin in A431 cells (data not shown). Silibinin also increased the number of both annexin V plus PI-stained cells accounting for 3.3 (control) to 4.3–7.5% and 3.9 (control) to 4.6–14.5%, after 48 and 72 h of treatment, respectively (Figure 6B). An apoptotic effect of silibinin on A431 cells at 24 h of treatment at these doses was less profound as compared with that at 48 and 72 h treatment (data not shown). Together, these results correlate well with silibinin-caused cell death data shown in Figure 5B, and also support the apoptotic nature of cell death caused by silymarin in skin tumors.

Physiological level of silibinin inhibits MAPK/ERK1/2 activation and up-regulates SAPK/JNK1/2 and p38 MAPK activation in A431 cells

In signal transduction pathways, MAPK family proteins have been regarded as a convergence point for extracellular signals, and have been implicated in multi-stage skin carcinogenesis (28,31). As a result of a strong growth inhibitory effect of silymarin on established skin tumors in mice and its association with an in vivo inhibition of MAPK/ERK1/2 activation, we next investigated the effect of silibinin on the constitutive activation status of MAPK family proteins in A431 cells. As shown in Figure 7A (upper panel), the treatment of A431 cells with silibinin at 12.5, 25 and 50 µM doses for 24 and 48 h resulted in a significant inhibition of ERK1/2 activation as evidenced by a decrease in the levels of phospho-p44 and -p42 proteins. Densitometric analysis of blots showed that compared with the control, 24 h of silibinin treatment at 12.5, 25 and 50 µM doses resulted in 52, 76 and 67% decrease in ERK1 phosphorylation, respectively (Figure 7B). In the case of ERK2 phosphorylation, these treatments were moderately effective and showed 22, 42 and 32% decrease, respectively (Figure 7B). As compared with 24 h treatment, the silibinin treatment for 48 h showed lesser inhibitory effect on MAPK/ERK1/2 activation (Figure 7A and B). The observed inhibitory effect of silibinin on ERK1/2 activation was not as result of a decrease in total ERK1/2 protein levels (Figure 7A, bottom panel), suggesting that silibinin impairs constitutive ERK1/2 signaling. Overall, the physiological concentration of silibinin (25 µM) from the animal study showed a strong inhibitory effect on ERK1/2 activation in A431 cells, which was consistent with its inhibitory effect on ERK1/2 activation in established skin tumors.

Next we assessed the effect of silibinin on SAPK/JNK1/2

4.4 µg of silybin/ml plasma or per gram tissue in plasma, skin tumor, skin, liver, lung, mammary gland and spleen, respectively. The plasma level of silibinin, and its half and twice doses were used in further studies detailed next to assess its anticancer effect in human epidermoid carcinoma A431 cells.

Physiological level of silibinin inhibits growth and causes death of A431 cells

Silibinin treatment of A431 cells resulted in a significant inhibition of their growth in both time- and dose-dependent manner. Silibinin treatment at 25 µM dose (physiological concentration achievable in animal study) for 1–3 days resulted in 49–59% (P < 0.001) inhibition of cell growth (Figure 5A). Even the lower dose (12.5 µM) of silibinin was effective in significantly inhibiting (21–36% inhibition, P < 0.01–0.001) cell growth after 1–3 days of treatment. The observed cell growth inhibition further increased to 70–74% (P < 0.001) at 50 µM dose of silibinin. In the cell death study, silibinin induced both time- and dose-dependent death of A431 cells. Compared with DMSO-treated controls showing 10–19% cell death during 1–3 days, silibinin treatment resulted in 19–35, 32–41 and 39–43% cell death after 1–3 days of treatment at 12.5, 25 and 50 µM doses, respectively (Figure 5B). Although it might not be relevant in terms of physiological levels, silibinin treatment at higher doses and longer treatment times almost completely inhibited cellular proliferation and caused more profound death of A431 cells (data not shown).

Physiological level of silibinin inhibits growth of established skin tumors
Fig. 6. Induction of apoptotic cell death by silibinin in A431 cells. Cells were treated with either DMSO or 12.5, 25 and 50 µM of silibinin for 48 or 72 h. After these treatments, cells were harvested and processed for annexin and PI staining as referred to in ‘Materials and methods’. Percent of annexin-stained cells as an early apoptotic response (A); and percent of annexin plus PI-stained cells (late apoptotic cells) (B) are from a representative experiment repeated twice with similar results: each treatment done in duplicate.

and p38 MAPK activation in A431 cells that may possibly be involved in apoptotic cell death. Similar silibinin treatments for 24 h resulted in a strong increase in the activation of JNK1 (up to 9-fold) and JNK2 (up to 5-fold) in a dose-dependent manner (Figure 8A and B), which diminished after 48 h of treatment. The observed effect of silibinin on JNK1/2 activation (Figure 8A, upper panel) was not due to an increase in total JNK1/2 protein levels (Figure 8A, bottom panel), suggesting that silibinin up-regulates SAPK/JNK signaling in A431 cells. p38 MAPK was also activated after 24 h of these silibinin treatments; however, after 48 h, only higher doses (50 µM) of silibinin showed an increase in p38 MAPK activation (Figure 8C and D). At 24 h of treatment, total p38 MAPK protein levels did not change but at 48 h of treatment, a decrease in protein level was noticed with increasing doses of silibinin (Figure 8C, lower panel). Overall, compared with p38 MAPK activation, silibinin-caused JNK1/2 activation was very prominent in A431 cells, which may be associated with increased apoptosis by silibinin.

Inhibition of MAPK/ERK1/2 is in part responsible for growth inhibition and death of A431 cells by silibinin

To further delineate whether inhibition of MAPK/ERK1/2 signaling by silibinin is the major, or in part, contributor to the inhibition of cell growth and induction of cell death by silibinin, we treated A431 cells with MEK1 inhibitor PD98059 (50 µM) and silibinin (50 µM) either alone or in combination for 24 h. In the MTT assay, silibinin and PD98059 decreased the cell viability by 43 (P < 0.05) and 46% (P < 0.01), respectively, but when the two were combined, cell viability decreased by 82% (P < 0.001) as compared with that of the control (Figure 9A). The decrease in cell viability in combination was statistically significant (P < 0.05–0.01) when compared with the effect of either compound alone. In the total cell count, also, silibinin and PD98059 showed similar trend in cell growth inhibition either alone (P < 0.01–0.001) or in combination (P < 0.001) (Figure 9B). Silibinin as well as silibinin in combination with PD98059 also showed increased cell death (data not shown). Together, the results suggest that whereas inhibition of MAPK/ERK1/2 activation by either silibinin or PD98059 is important, it is contributory only in part for the observed growth inhibition and death of A431 cells. Other mechanisms such as activation of SAPK/JNK1/2 and p38 MAPK pathways, possibly also contribute significantly to the observed biological responses by silibinin.

Discussion

The central finding of the present study is that naturally occurring flavonoid antioxidant silymarin strongly inhibits established skin tumor growth accompanied by tumor regression which is associated with a decrease in proliferation index together with an inhibition of MAPK/ERK1/2 signaling.
Silymarin inhibits growth of established skin tumors

Fig. 8. Silibinin increases the activation of SAPK/JNK1/2 and p38 MAPK in A431 cells. Silibinin treatments, culture conditions, SDS–PAGE and western blotting were the same as in Figure 7. The membranes were probed with anti-phospho-JNK1/2, regular JNK1/2, phospho-p38 and regular p38 antibodies and then with peroxidase-conjugated appropriate secondary antibody. Proteins were visualized with ECL detection system. (A) Phosphorylation of JNK1/2 (upper panel), total levels of JNK1/2 (bottom panel) and (B) densitometric analysis for phospho-JNK1 and phospho-JNK2. (C) Phosphorylation of p38 (upper panel), total levels of p38 (bottom panel) and (D) densitometric analysis for phospho-p38. For densitometric analysis, blots were corrected for loading with the density of JNK1/2 and p38 blots. The experiment was repeated twice with similar results and treatments were as labeled in the figure.

Fig. 9. Effect of MEK1 inhibitor (PD98059) and silibinin on cell viability and growth of A431 cells. For MTT assay, 5000 cells/well were plated in 96 well plate and treated either with vehicle (0.1% DMSO v/v) or with silibinin (50 µM), PD98059 (50 µM) or both together for 24 h. At the end of the treatment, cells were processed as described in ‘Materials and methods’, and absorbance was read at 590 nm against DMSO (A). Similar silibinin and PD98059 doses were also used for cell growth analysis (B) as described in ‘Materials and methods’. In each case, the data shown are mean ± SE of three to four samples. Experiments were repeated with similar results.

and additional molecular and genetic events lead to enhanced mitogenic and cell survival signaling during promotion and progression stages (32–34). The ras oncogene is dysregulated and/or mutated frequently in several human malignancies making them unresponsive to standard treatments (35,36). This makes the ras/raf/MEK/ERK pathway a potential target for the therapeutic intervention of various human cancers. Our understanding of mitogenic signaling and apoptotic pathways has yielded new strategies to prevent and/or intervene the process of tumor development by pharmacological methods as well as nutritional intervention (37). An enhanced level of growth factors and their receptors and persistent MAPK signaling via enhanced maturation of mutated Ha-ras oncogene product, ras p21, has been implicated in skin tumorigenesis (38,39). Ras activation leads to the sequential activation of Ser/Thr kinase Raf-1, MEK1/2 and ERK1/2 (40). MAPKs are highly conserved and act as second messengers in transducing the extracellular signals to the intracellular milieu for appropriate responses such as cell proliferation, differentiation, inflammation, malignant transformation and apoptosis (41-42 and references therein). ERK1/2 are activated by a variety of extracellular signals including mitogens and contribute to the proliferative responses in cells, and are considered to be an
essential common element of mitogenic signaling (41–43). Their constitutive expression causes cell transformation and plays a putative role in the carcinogenesis process (43–46). Accordingly, the observed effect of silymarin on tumor cell growth inhibition and apoptosis induction under both in vivo and in vitro systems together with a decrease in phospho-ERK1/2 levels suggest a possibility that an initial response silymarin modulates ERK1/2 activation that leads to inhibition of tumor growth and regression by apoptotic cell death.

During mammalian cell growth and proliferation, in most cell types, the mitogenic signals are transduced to the nucleus by the nuclear translocation of ERK1 and 2, resulting in the activation of various transcription factors including c-Myc and Elk1 (47–49). Recent studies have shown that these Ser/Thr protein kinases play a determinant role in the carcinogenesis process and their activation is sufficient to stimulate early gene transcription and to reduce growth factor requirement for DNA synthesis (47,49). Enhanced expression of PCNA, a 36 kDa co-factor of DNA polymerase δ, is one of the downstream effects of the activation of MAPK/ERK1/2 signaling and well correlated to the status of cell proliferation. PCNA is a potentially useful molecular biomarker of cellular proliferation kinetics and fits to the expected biological mechanisms where it can be correlated to the decreased/increased cancer incidence (50). Based on a series of epidemiological and experimental studies in recent years, it is clearly evident that changes in dietary and/or nutritional patterns might have profound and immediate impact on reducing the cancer incidence. Accordingly, the results of our present study, showing strong growth inhibition of established skin tumors accompanying significant decrease in proliferation index and inhibition of MAPK/ERK1/2 activation, and similar anticancer effects of silibinin on human epidermoid carcinoma A431 cells via similar mechanism, provide a potential strategy of nutritional intervention of skin cancer. SAPK/JNK1/2 and p38 MAPK signal transduction pathways have been shown to mediate various forms of cellular stress, such as damage repair mechanisms, cell growth arrest and apoptotic cell death (51,52).

It has been demonstrated that the activation of JNK and p38 with concurrent inhibition of ERK is critical for the induction of apoptosis in abnormal cells including cancer cells (53 and references therein). Therefore, the results of our present investigation showing inhibition of ERK activation and simultaneous increase in JNK and p38 activation, suggest the involvement of these pathways in silibinin-caused apoptosis in A431 cells, and that similar mechanisms may also be operative in in vivo conditions. Additional studies are needed to support this suggestion, and are in fact the major focus of our ongoing work in this area. As recent clinical trials have demonstrated the potential of signal transduction modulators in cancer treatment (54 and references therein), our present findings demonstrate the strong potential of silymarin for targeting both mitogenic and survival pathways in cancer cells.

The present data combined with our earlier studies suggest that more mechanistic studies are needed to further substantiate the therapeutic efficacy of silymarin or silibinin against human cancers. The induction of apoptotic cell death was identified as another possible anticarcinogenic mechanism of silymarin against skin cancer. The results from TUNEL analysis of skin tumor samples, and annexin V and PI staining of A431 cells showed that apoptosis predominantly contributes to tumor cell killing caused by silymarin or silibinin. It is well established that apoptosis and, the associated cell-signaling pathways and cellular events controlling it, have a profound effect on the progression of benign to malignant phenotype, and that they can be targeted for the therapy of various malignancies including skin cancer (55–57). The molecular mechanism of apoptosis induction in skin tumor by silymarin is yet to be explored. Studies are in progress to identify the signaling pathways and molecular events associated with silymarin-caused apoptotic cell death in skin tumor under both in vivo and cell culture conditions.

In the present study, we also observed that dietary administration of silymarin results in its distribution in several important organs of the body, which are generally susceptible to cancer development. These systemic bio-distribution results of silymarin, and the fact that tissue specificity and histopathological course of tumor development in mice is remarkably similar to that observed in several human epithelial malignancies (58), suggest that silymarin could be a novel compound for both prevention (based on earlier studies) and therapy of different epithelial cancers. This suggestion could at least in part be supported by a series of recent cell culture studies showing an anticancer potential of silymarin and/or silibinin against human skin, prostate, breast and cervical cancers (28,59–61).

In summary, based on the results of the present study and those reported recently, investigational clinical trials with collaborative laboratory studies are needed to develop silymarin as both preventive and therapeutic agent against skin and other epithelial cancers in humans. A positive outcome of such studies could specifically be beneficial as silymarin is already in clinical use as an anti-hepatotoxic agent and consumed as a dietary supplement around the world including the USA, and is devoid of any untoward toxicity and side effects.

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Silymarin inhibits growth of established skin tumors

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