A Flavonoid Antioxidant, Silymarin, Affords Exceptionally High Protection against Tumor Promotion in the SENCAR Mouse Skin Tumorigenesis Model

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ABSTRACT

In cancer chemoprevention studies, the identification of better antitumor-promoting agents is highly desired because they may have a wider applicability against the development of clinical cancers. Both epidemiological and animal studies have suggested that microchemicals present in the diet and several herbs and plants with diversified pharmacological properties are useful agents for the prevention of a wide variety of human cancers. Silymarin, a flavonoid isolated from milk thistle, is used clinically in Europe and Asia as an antihepatotoxic agent, largely due to its strong antioxidant activity. Because most antioxidants afford protection against tumor promotion, in this study, we assessed the protective effect of silymarin on tumor promotion in the SENCAR mouse skin tumorigenesis model. Application of silymarin prior to each 12-O-tetradecanoylphorbol 13-acetate (TPA) application resulted in a highly significant protection against tumor promotion in 7,12-dimethylbenz(a)anthracene-initiated mouse skin. The protective effect of silymarin was evident in terms of reduction in tumor incidence (25, 40, and 75% protection, \(P < 0.001\), \(X^2\) test), tumor multiplicity (76, 84, and 97% protection, \(P < 0.001\), Wilcoxon rank sum test), and tumor volume (76, 94, and 96% protection, \(P < 0.001\), Student's \(t\) test) at the doses of 3, 6, and 12 mg per application, respectively. To dissect out the stage specificity of silymarin against tumor promotion, we next assessed its effect against both stage I and stage II of tumor promotion. Application of silymarin prior to that of TPA in stage I or mezerein in stage II tumor promotion in dimethylbenz(a)anthracene-initiated SENCAR mouse skin resulted in an exceptionally high protective effect during stage I tumor promotion, showing 74% protection against tumor incidence (\(P < 0.001\), \(X^2\) test), 92% protection against tumor multiplicity (\(P < 0.001\), Wilcoxon rank sum test), and 96% protection against tumor volume (\(P < 0.001\), Student's \(t\) test). With regard to stage II tumor promotion, silymarin showed 26, 63, and 54% protection in tumor incidence, multiplicity, and volume, respectively. Similar effect of silymarin to that in anti-stage I studies, were also observed when applied during both stage I and stage II protocols. In other studies, silymarin significantly inhibited: (a) TPA-induced skin edema, epidermal hyperplasia, and proliferating cell nuclear antigen-positive cells; (b) DNA synthesis; and (c) epidermal lipid peroxidation, the early markers of TPA-caused changes that are associated with tumor promotion. Taken together, these results suggest that silymarin possesses exceptionally high protective effects against tumor promotion, primarily targeted against stage I tumors, and that the mechanism of such effects may involve inhibition of promoter-induced edema, hyperplasia, proliferation index, and oxidant state.

INTRODUCTION

Cancer is the third major cause of mortality, accounting for more than 7 million deaths per year worldwide (1). The major goal of cancer research, therefore, has been to achieve an understanding of the processes involved in the induction followed by development of human cancers, which could allow diagnosis, an early detection, and therapy as well as prevention of this disease. Indeed, recent developments and advancements in first three aspects have been excellent, improving, at least in part, prognosis and quality of life for cancer patients. However, there is still little improvement in the mortality rates for most cancers in general and, in particular, for a subpopulation of cancer patients who are at a higher-than-average risk. In view of these and other limitations, increasing attention has also been focused on preventing the occurrence of cancer incidence in the first place. Chemoprevention of cancer, therefore, is a means of cancer control by which the rate of this disease can be prevented totally or slowed or reversed partially or substantially by the administration of one or more naturally occurring or synthetic chemical agents. Fruits, vegetables, and common beverages as well as several herbs and plants with diversified pharmacological properties have been shown to be rich sources of microchemicals with the potential to prevent human cancers (Refs. 2–10 and references therein). Measuring the effects of these agents in cancer chemoprevention studies in human populations has now become one important objective of experimental cancer research. The potential for inhibiting tumor development in both targeted high-risk and general populations has increased significantly in recent years (2–10). Accordingly, many new classes of chemical compounds are being evaluated in clinical trials as cancer-preventive agents for several malignancies (2–10).

About 30 classes of chemicals with cancer-preventive effects that may have practical implications in reducing cancer incidence in human population have been described (11). Among these, naturally occurring polyphenolic antioxidants are receiving increasing attention in recent years (Refs. 12–14 and references therein). Using different long-term experimental tumorigenesis protocols, several studies have demonstrated the cancer-preventive effects of polyphenolic antioxidants (12–14). Silymarin is also a polyphenolic flavonoid antioxidant isolated from milk thistle [Silybum marianum (L.) Gaertn; Ref. 15]. It is being used clinically in Europe and Asia for the treatment of alcoholic liver diseases (16, 17). As a therapeutic agent, silymarin is well tolerated and largely free of adverse effects (Refs. 18 and 19 and references therein), so much so that silymarin has also been marketed recently in the United States and Europe as a nutritional supplement by several “nutraceutical companies.” Studies on mice, rats, rabbits, and dogs, using different modes of administration, showed that silymarin is nontoxic in both acute and chronic tests and does not show any side effects (20); there is no known LD\(_{50}\) for silymarin in laboratory animals (19–22). Several studies in rodents have shown that silymarin protects against hepatotoxicity induced by allyl alcohol, carbon tetrachloride, galactosamine, phalloidin, thioacetamide, and microcystin-LR (23, 24). Mechanistic studies have shown that silymarin is a strong antioxidant that is capable of scavenging both free radicals and reactive oxygen species in rodents and in cell cultures and that it results in a significant increase in cellular antioxidant defense machinery by ameliorating the deleterious effects of free radical reactions (Refs. 25–28 and references therein). Other studies have also shown that silymarin inhibits the formation of transformed rat tracheal epithelial cell colonies induced by exposure to benzo(a)pyrene (29),...
TPA\(^2\)-induced anchorage-independent growth of JB6 mouse epidermal cells (30), and DMBA-initiated and TPA-promoted mammary lesion formation in organ culture (31).

Studies performed recently in our laboratory have shown that silymarin almost completely inhibits TPA-induced epidermal ODC activity and mRNA expression and also significantly inhibits epidermal ODC activity induced by structurally different skin tumor promoters, including free radical-generating compounds (32). More recently, we also showed that topical application of silymarin on to the mouse skin results in a highly significant protection against UVB radiation- and chemical-induced tumorigenesis in mouse skin (33, 34). Mechanistic studies defining the protective effect of silymarin on chemical tumor promoter-caused skin tumor promotion in DMBA-initiated SENCAR mice showed that its application prior to TPA and okadaic acid resulted in a highly significant complete inhibition of the epidermal TNF-\(\alpha\) mRNA expression induced by these two skin tumor promoters, in a dose-dependent manner (34); TNF-\(\alpha\) has also been implicated as an endogenous tumor promoter involved in mouse skin tumor promotion process (35). Taken together, on the basis of these findings, here we performed a dose seeking study assessing the protective effect of silymarin against tumor promotion in SENCAR mouse skin tumorigenesis model. It is important to emphasize here that the promotion stage of multistage mouse skin carcinogenesis exists in two operationally distinct stages known as stage I and stage II tumor promotion (36–40). Stage I tumor promotion can be effectively achieved by limited applications of the potent tumor promoter TPA but is insufficient to produce tumors in initiated mouse skin (36–40). MEZ, a diterpene that is similar to TPA in its biochemical and morphological effects but a weak and incomplete tumor promoter, is a potent stage II promoter when given repetitively after stage I tumor promotion (37–40). Additional studies, therefore, were also performed to dissect out whether silymarin is a stage-specific inhibitor of tumor promotion in mouse skin tumorigenesis model in which TPA and MEZ are used as stage I and stage II tumor promoters, respectively.

In the mouse skin tumorigenesis model, the promotion stage is achieved by repeated applications of tumor promoter following the tumor initiation with the subcarcinogenic dose of an initiating agent (38–43). The tumor promotion stage has been studied most thoroughly with respect to the phorbol ester tumor promoters that produce numerous histological and biochemical changes in mouse skin (38–43). Specifically, the topical application of TPA to mouse skin is shown to result in a number of biochemical alterations, changes in cellular functions, and histological changes leading to skin tumor promotion (Refs. 38–43 and references therein). Among these, the ones that best correlate with skin tumor-promoting activity of TPA include skin edema; epidermal hyperplasia; inflammation; increase in the number of dark basal keratinocytes with PCNA staining, suggesting proliferation; neutrophil infiltration; induction of epidermal lipoygenase; cyclooxygenase-dependent metabolism of arachidonic acid; oxidative stress causing increased hydrogen peroxide formation and enhanced lipid peroxidation, and so on (38–43). All these changes in skin have been defined as possible markers of stage I of skin tumor promotion (38–43). Following TPA application of mouse skin, the elevated levels of epidermal ODC, polyamines, protein kinase C, and so on are the events that have been related to the stage II of skin tumor promotion (38–43). It is however, difficult to determine which of these or the many other effects of the phorbol esters are sufficient components of the promotion process. Whereas earlier studies from our laboratory have shown the inhibitory effect of silymarin on TPA-induced epidermal ODC activity and mRNA expression in SENCAR mice (32), studies were performed in this paper to assess whether silymarin also inhibits short-term markers associated with early effects of topically applied TPA on mouse skin during stage I of tumor promotion.

### MATERIALS AND METHODS

**Chemicals.** TPA, MEZ, and DMBA were purchased from Sigma Chemical Co. (St. Louis, MO), and silymarin was from Aldrich Chemical Co. (Milwaukee, WI). All other chemicals were obtained in the purest form available commercially.

**Animals.** Six-week-old female SENCAR mice were obtained from Harlan Sprague Dawley (Indianapolis, IN). The animals were housed five per cage at 24 ± 2°C and 50 ± 10% relative humidity and subjected to a 12-h light/12-h dark cycle. They were acclimatized for 1 week before use and fed a Purina Chow diet and water ad libitum. Prior to any study, the dorsal side of the skin was shaved using electric clippers, and the mice with hair cycle in the resting phase were used in all of the studies.

**Skin Tumor Promotion Study.** The animals were randomly divided into six groups of 20 animals each, and a single 10-nmol dose of DMBA per mouse was applied topically on the dorsal shaved skin of mice in groups I–IV in 0.2 ml of acetone as tumor initiator. One week later, animals in group I were treated topically with 0.2 ml of acetone, and animals in groups II, III, and IV were treated with 3-, 6-, and 12-mg doses of silymarin per mouse per application, respectively, in 0.2 ml of acetone. Thirty min after these treatments, a 3.2-nmol dose of TPA per mouse per application was applied topically to animals in groups I–IV. The pretreatment regimen for silymarin was based on our study showing that topical application of silymarin 30 min prior to that of TPA produces maximum inhibitory effect against TPA-induced epidermal ODC activity and mRNA expression (32). The TPA alone or various doses of silymarin plus TPA treatments were repeated two times per week up to the termination of the experiment at 20 weeks from the start of DMBA. The animals in group V were treated with 0.2 ml of acetone alone and served as a negative control for any spontaneous tumor induction. To assess whether silymarin alone produces tumor promoting effects, we initiated the animals in group VI with a 10-nmol dose of DMBA and, 1 week later, treated them with a 12-mg dose of silymarin per mouse per application twice a week until the end of the experiment at 20 weeks. Animals in all of the groups were watched for food and water consumption and any apparent signs of toxicity, such as weight loss or mortality, during the entire period of the study. Skin tumor formation was recorded weekly, and tumors of >1 mm in diameter were included in the cumulative total if they persisted for 2 weeks or more. Latent periods for the onset of tumor in various groups were computed, and the tumors were diagnosed histologically at the termination of the experiment. At this point, the total tumor volume on the back of each mouse was also recorded.

**Stage-specific Skin Tumor Promotion Studies.** The animals were randomly divided into four groups of 20 animals each, and a single 10-nmol dose of DMBA per mouse in 0.2 ml of acetone as tumor initiator was applied topically on the dorsal shaved skin. One week after initiation, stage I and stage II tumor promotion was achieved by topical application of TPA and MEZ, respectively, and stage-specific preventive effects of silymarin were assessed by its application prior to TPA, MEZ, or both. Following tumor initiation with DMBA, the four experimental groups were: group 1, TPA (stage I) plus MEZ (stage II); group 2, [6-mg dose of silymarin plus TPA (anti-stage I)] plus MEZ (stage II); group 3, TPA (stage I) plus [6-mg dose of silymarin plus MEZ (anti-stage I)]; and group 4, [6-mg dose of silymarin plus TPA (anti-stage I)] plus [6-mg dose of silymarin plus MEZ (anti-stage II)]. To achieve stage I tumor promotion, the animals were treated twice a week for 4 weeks with a 3.2-nmol dose of TPA. For the remaining period of the experiment, mice were treated topically twice a week with 3.2-nmol dose of MEZ for stage II tumor promotion. In each case, silymarin was applied topically to the shaved areas of individual mice 30 min prior to each application of either TPA or MEZ (at stage I or stage II, respectively) or both (at stage I and stage II). The selection of the 6-mg dose of silymarin was based on complete tumor promotion studies in which this dose resulted in a highly significant protection against TPA-caused tumor promotion in mouse skin (see “Results”). The doses and treatment regimen for TPA and MEZ during stage I and stage II tumor promotion were based on those reported previously (44, 45). For each treatment, every compound was dissolved in 0.2 ml of acetone per application. Animals in all
of the groups were watched for food and water consumption, and any apparent signs of toxicity such as weight loss or mortality during the entire period of the study. Skin tumor formation was recorded weekly, and tumors of >1 mm in diameter were included in the cumulative total if they persisted for 2 weeks or more. Latent periods for the onset of tumor in various groups were computed, and the tumors were diagnosed histologically at the termination of the experiment at 24 weeks from the start of DMBA application. At this point, the total tumor volume on the back of each mice was also recorded.

**Skin Edema and Epidermal Hyperplasia Studies.** The animals were divided into several groups with three mice in each and treated topically on the shaved area with 0.2 ml of acetone, 6 mg of silymarin in 0.2 ml of acetone, 5 nmol of TPA in 0.2 ml of acetone, or 6 mg of silymarin in 0.2 ml of acetone followed 30 min later with 5 nmol of TPA in 0.2 ml of acetone. These treatments were performed once, twice (48 h apart), or three times (24 h apart), and 24 h after the last treatment, animals were sacrificed and the treated area of the skin was used for the desired studies.

To assess the extent of TPA-caused edema in SENCAR mouse skin and the inhibitory effect of preapplication of silymarin on TPA-caused skin edema, we removed 1-cm-diameter punches of skin, removed the fat pads, and quickly weighed the samples. After drying for 24 h at 50°C, the skin punches were reweighed, and the loss of water content was determined. The difference in the amount of water gain between acetone-treated negative controls and TPA-treated positive control represented the extent of skin edema by TPA, whereas the ratio between acetone control and silymarin plus TPA represented the inhibitory effect of silymarin.

For the epidermal hyperplasia study, skin samples from different treatment groups were fixed in 10% formalin and embedded in paraffin. Vertical sections (5 μm) were cut, mounted on a glass slide, and stained with H&E. Epidermal hyperplasia was determined as mean vertical epidermal thickness and mean number of vertical epidermal cell layers by microscopic examination of different treated skin tissue sections. For each section of the skin, the thickness of the epidermis from the basal layer to stratum corneum was measured at several equal distance interfollicular sites using a Zeiss light microscope (Germany) equipped with an ocular micrometer. For each individual value, a mean of 25 vertical epidermal measurements, equidistant from each other at 2 mm, in 50 mm (linear) of skin section was determined.

**PCNA Immunohistochemical Staining Study.** Tissue sections obtained from different treated skin specimens (those used for H&E staining) were deparaffinized using xylene and rehydrated in a graded series of alcohol with a final wash in distilled water. Sections were heated in microwave two times for 4 min each in 10 nm citrate buffer (pH 6.0), cooled to room temperature, and incubated in 10% blocking solution [100% horse serum diluted in 10 nm PBS (pH 7.4), v/v] for 15 min at 37°C in a humidity chamber. The sections were then incubated with mouse monoclonal anti-PCNA antibody IgG2a (1:1800 dilution in 10% blocking solution) for 1 h at 37°C in a humidity chamber. Negative controls were incubated with p53 antibody IgG2a as an inappropriate antibody (1:454 dilution in 10% blocking solution) under identical conditions. The sections were rinsed with 10 nm PBS and then incubated with biotinylated antimouse antibody IgG (1:400 dilution in 10% blocking solution) for 30 min at room temperature. Thereafter, the sections were washed with 10 nm PBS, incubated with methanol, hydrogen peroxide, and water solution (30:0.3:69.7, v/v/v) for 10 min at room temperature and then rinsed with distilled water. The sections were then incubated with streptavidin-biotinylated horseradish peroxidase (1:100 dilution in 10% blocking solution) for 30 min at room temperature in humidity chamber followed by three 3-min washings with 10 nm PBS. The sections were then incubated with 3,3'-diaminobenzidine solution for 20 min at room temperature followed by washing with distilled water and counterstaining with 0.1% methyl green for 20 s. The slides were then dehydrated, mounted, viewed, and photographed. The quantitation of proliferating cells was made by counting the PCNA-positive cells as well as the total cells at 10 arbitrarily selected fields at ×40 magnification within the epidermis and the interfollicular regions in a double-blinded manner. The percentage of PCNA-positive cells per 10 × 40 fields for different treatment samples was determined as: number of PCNA-positive cells × 100/total number of cells.

**DNA Synthesis Study.** The effect of silymarin on DNA synthesis was assessed by measuring [1H]thymidine incorporation using human epidermoid carcinoma A431 cells obtained from the American Type Culture Collection (Manassas, VA). The cells were seeded at a density of 1000 cells in 0.2 ml of DMEM with 10% FCS and 1% penicillin-streptomycin per well in a 96-well microtiter plate and left overnight at 37°C in an atmosphere of 95% air and 5% CO2. The next day, fresh medium, containing either ethanol alone or varying concentrations (7.5, 15, and 30 μM final concentrations) of silymarin dissolved as stock in ethanol and diluted in medium, was added to the cultures. In no case was the final concentration of ethanol >0.5% of the medium. The cells were incubated for 4 h at 37°C and then labeled with 1 μCi of [methyl-3H]thymidine (83 Ci/mmol; DuPont NEN, Boston, MA) per well for 18 h at 37°C. The cells were harvested thereafter on filter mats using a cell harvester, and the filters were washed extensively once with water followed by ethanol. The cell-bound radioactivity (on filter mats) was determined by liquid scintillation counting.

**Lipid Peroxidation Study.** SENCAR mice were sacrificed by cervical dislocation, dorsal shaved skin was excised, and epidermal microsomes were prepared as described previously (46). The microsomes were washed with and suspended in 0.1 M phosphate buffer (pH 7.4) containing 0.1 mM MgCl2. The generation of MDA was used as a marker of lipid peroxidation and estimated by the method of Wright et al. (47) with some modifications described previously (48). The final concentration of MDA was calculated using a molar extinction coefficient of 1.56 × 105 M−1 cm−1. For those incubations which involved silymarin, it was dissolved in acetone and added to the incubation mixture prior to the start of incubation; the final concentrations of silymarin used were between 0.1 and 20 μM. The inhibition of lipid peroxidation by silymarin was determined by measuring the reduction in the amount of MDA in silymarin-added incubations compared to acetone alone controls.

**Statistical Analysis.** In tumorigenesis experiments, the statistical significance of differences in terms of tumor incidence and multiplicity between TPA and silymarin plus TPA and between TPA plus MEZ and silymarin plus TPA plus MEZ groups was evaluated by χ2 and Wilcoxon rank sum tests, respectively. For tumor volume data and all other short-term studies, Student’s t test was used to assess the statistical significance of difference.

**RESULTS**

**Preventive Effect of Silymarin against Tumor Promotion.** Topical application of silymarin prior to each TPA application resulted in a highly significant preventive effect against TPA-caused complete tumor promotion in DMBA-initiated SENCAR mouse skin. The preventive effect of silymarin was dose dependent and was evident by a highly significant reduction in tumor incidence, tumor multiplicity, and tumor volume (Fig. 1 and Table 1). In terms of any toxic effects of topical application of silymarin, as monitored by weight gain profile, no noticeable difference was observed between varying doses of silymarin-treated and non-silymarin-treated groups of animals throughout the experiment (Fig. 1A). In addition, the mean water consumption per animal per day was also comparable between varying doses of silymarin-treated and non-silymarin-treated group of animals (data not shown). Taken together, these observations suggest that topical application of silymarin, at least up to 12 mg dose applied twice weekly, does not produce any apparent toxicity during the entire period of the experiment.

In terms of anti-tumor promotion results, when the data were analyzed for tumor incidence, as shown in Fig. 1B, topical application of silymarin prior to that of TPA in DMBA-initiated SENCAR mouse skin resulted in a dose-dependent protection throughout the experiment. Compared to the non-silymarin-treated positive control group of mice, the time of appearance of first tumor was delayed by 1–2 weeks in silymarin-treated animals. When these data were assessed at the middle of the experiment at 10 weeks (Fig. 1B), compared to 100% mice with skin tumors in non-silymarin-treated group, only 5% of animals in the 12-mg silymarin-treated group and only 20% of animals in the 3- and 6-mg silymarin-treated groups developed tumors (P < 0.001, χ2 test). At the termination of the experiment at 20 weeks, compared to 100% animals with skin tumors in the non-silymarin-treated positive control group, only 75, 60, and 25% of the animals in the 3-, 6-, and 12-mg silymarin-treated groups exhibited skin neoplasms accounting for 25, 40, and 75% protection (P < 0.001, χ2 test) in tumor incidence, respectively (Fig. 1B).
Similarly, when the tumor data were evaluated for tumor multiplicity (cumulative number of tumors per group or number of tumors per mouse), beginning with the first tumor appearance up to the termination of the experiment, all of the three doses of silymarin used in this study produced highly significant protection against TPA-caused complete tumor promotion in mouse skin (Fig. 1C). At the termination of the experiment, compared to 17 ± 3 (mean ± SE of 20 mice) tumors per mouse in non-silymarin-treated group, only 4.15 ± 1.10, 2.65 ± 0.56, and 0.55 ± 0.09 (mean ± SE of 20 mice in each case) tumors per mouse were observed in the 3-, 6-, and 12-mg silymarin-treated groups, respectively, accounting for 76, 84, and 97% protection ($P < 0.001$, Wilcoxon rank sum test), respectively (Fig. 1C).

When the tumor promotion data were analyzed in terms of tumor volume, compared to non-silymarin-treated positive control group, the total tumor volume and tumor volume per mouse were found to be significantly lower (76–96%; $P < 0.001$, Student’s $t$ test) in different doses of silymarin-treated groups (Table 1). However, only the 6-mg dose of the silymarin-treated group of mice also showed a significant decrease (61%; $P < 0.001$, Student’s $t$ test) in tumor volume per tumor (Table 1). The tumors in each group of mice were histologically identified as benign papillomas (data not shown). The animals initiated with DMBA and promoted twice weekly with a 12-mg dose of silymarin were devoid of any skin tumors throughout the experiment (data not shown), suggesting that silymarin alone is not a tumor promoter.

**Preventive Effect of Silymarin against Stage-specific Tumor Promotion.** On the basis of the results of complete tumor promotion studies, additional experiments were performed to assess whether the preventive effect of silymarin against tumor promotion in mouse skin is a stage-specific phenomenon. As shown by data in Fig. 2 and Table 1, topical application of silymarin prior to TPA in stage I or MEZ in stage II resulted in an exceptionally high preventive effect in stage I tumor promotion in mouse skin. Much more profound preventive effect of silymarin, however, was observed when it was applied prior to tumor promoters in both stage I and stage II of mouse skin tumor promotion (Fig. 2). Similar to complete tumor promotion studies, no significant change was observed in the body weight gain profile between silymarin and non-silymarin treated groups of mice throughout the experiment (Fig. 2A), which further supported the previous observation that silymarin applied topically has no apparent toxicity.

In terms of tumor incidence, the exceptionally high preventive effect of silymarin against stage I tumor promotion was evident throughout the experiment (Fig. 2B). However, when these data were analyzed at the termination of the experiment at 24 weeks, compared to non-silymarin-treated positive controls showing 95% mice with skin tumors, application of silymarin in stage I tumor promotion resulted in only 25% mice with tumors accounting for 76% protection ($P < 0.001$, $X^2$ test) in tumor incidence (Fig. 2B). More strong preventive effects of silymarin were evident when it was applied during both stage I and II tumor promotion: only 15% mice developed skin tumors, an 84% reduction ($P < 0.001$, $X^2$ test) in tumor incidence (Fig. 2B). However, treatment of silymarin in stage II tumor promotion resulted in 70% of mice having skin tumors, representing only 26% protection (Fig. 2B). For the reasons not known, one mouse of a group of 20 animals in positive control group did not develop any skin tumors throughout the protocol, and therefore, this group accounted for 95% tumor incidence (Fig. 2B). When the preventive effects of silymarin were analyzed for delay in latency period on the onset of first tumor, compared to positive control group, a 5-week delay in the latency period on the onset of first tumor was observed in animals treated with silymarin prior to only TPA (anti-stage I) or both TPA and MEZ (anti-stage I and II; Fig. 2B). However, the onset of first tumor was delayed by only one week in animals treated with silymarin prior to MEZ (anti-stage II).

**Materials and Methods.** 1 week after topical application of 10 nmol of DMBA per mouse as tumor initiator, TPA was applied two times per week at the dose of 3.2 nmol per mouse without or with prior application of silymarin at doses of 3, 6, and 12 mg per mouse. These treatments were continued up to the end of the experiment at 20 weeks from the start of DMBA. The body weight (g) per mouse (A), percentage of mice with tumors (B), and number of tumors per mouse (C) were plotted as a function of the number of weeks on test. The body weight gain profile data shown are mean ± SE of 20 mice per group throughout the experiment. Similarly, the percentage of mice with tumor data are from 20 mice per group. The tumors per mouse data are also mean ± SE of 20 mice per group.

![Fig. 1. Dose-dependent preventive effect of topical application of silymarin on TPA-caused complete skin tumor promotion in DMBA-initiated SENCAR mice. As detailed in “Materials and Methods,” 1 week after topical application of 10 nmol of DMBA per mouse as tumor initiator, TPA was applied two times per week at the dose of 3.2 nmol per mouse without or with prior application of silymarin at doses of 3, 6, and 12 mg per mouse. These treatments were continued up to the end of the experiment at 20 weeks from the start of DMBA. The body weight (g) per mouse (A), percentage of mice with tumors (B), and number of tumors per mouse (C) were plotted as a function of the number of weeks on test. The body weight gain profile data shown are mean ± SE of 20 mice per group throughout the experiment. Similarly, the percentage of mice with tumor data are from 20 mice per group. The tumors per mouse data are also mean ± SE of 20 mice per group.](image-url)
Similar to tumor incidence data, preaplication of silymarin during stage I skin tumor promotion also showed highly significant protection against tumor multiplicity throughout the experimental protocol (Fig. 2C). At the termination of the experiment at 24 weeks, compared to 10.78 ± 1.72 (mean ± SE of 18 mice) tumors per mouse in the non-silymarin-treated positive control group, silymarin treatment in stage I promotion resulted in only 0.9 ± 0.48 (mean ± SE of 20 mice) tumor per mouse, representing 92% reduction (P < 0.001, Wilcoxon rank sum test; Fig. 2C). A more profound protective effect of silymarin in terms of tumor multiplicity was observed when it was applied during both stage I and stage II tumor promotion. In this protocol, compared to positive control group, only 0.15 ± 0.08 (mean ± SE of 20 mice) tumor per mouse was evident in silymarin-treated mice, representing a 99% reduction in tumor multiplicity (P < 0.001, Wilcoxon rank sum test; Fig. 2C). Parallel to its effect on tumor incidence, the protective effect of silymarin on tumor multiplicity was also less profound (compared to stage I and both stage I and II), although statistically significant (P < 0.001, Wilcoxon rank sum test), when applied during stage II skin tumor promotion. In this case, compared to positive control group, 63% reduction [3.95 ± 1.00 tumors per mouse (mean ± SE of twenty mice)] in tumor multiplicity was observed at the end of the experiment in silymarin-treated mice (Fig. 2C).

Interestingly, the inhibition in tumor multiplicity by silymarin in the three tumor promotion stage-specific protocols also corroborated with its inhibitory effect on total tumor volume as well as tumor volume per mouse in these protocols (Table 1). Animals in the anti-stage I and anti-stage I and II protocols showed 96 and 98% reduction (P < 0.001 in both cases, Student’s t test), respectively, in tumor volume per mouse, as compared to the non-silymarin-treated positive control group (Table 1). However, animals belonging to the anti-stage II group showed a 54% inhibition (P < 0.001, Student’s t test) in tumor volume per mouse. Whereas silymarin also showed a 52% reduction (P < 0.001, Student’s t test) in tumor volume per tumor in case of anti-stage I protocol (Table 1), no change was observed in anti-stage II and anti-stage I and II protocols (Table 1). It is important to mention here that, in the positive control group, the observed decrease in number of tumors per mouse beginning week 18 of treatment was not due to tumor regression (Fig. 2C). The decrease in this number from 13.33 ± 2.68 (mean ± SE of 18 mice) tumors per mouse at week 18 to 10.78 ± 1.72 (mean ± SE of 18 mice) tumors per mouse at the end of the experiment at week 24 was due to a significant increase in tumor size, which made these tumors to overlap each other (tumor volume data as a function of week tested are not shown).

**Inhibitory Effects of Silymarin on TPA-caused Skin Edema and Induction of Epidermal Hyperplasia.** To assess the inhibitory effect of silymarin on TPA-caused skin edema in SENCAR mice, three different experimental protocols were followed that used either single or multiple applications of TPA on to the shaved dorsal skin of mice without or with prior application of silymarin, and at a desired time thereafter, the weight of 1-cm-diameter punch biopsies of the treated skin was determined. As shown by data in Table 2, compared to acetone-treated negative control group, a single topical application of TPA on to the mouse skin resulted in a significant increase in skin edema in SENCAR mice. An additional topical application of TPA after 48 h at first treatment showed an additional increase in skin edema after 24 h of last treatment (Table 2). More profound TPA-caused skin edema in SENCAR mice was evident when it was applied topically for 3 consecutive days at 24-h time gaps (Table 2). Together, these findings were in accord with earlier studies showing the effect of acute and chronic treatment of mouse skin with TPA on skin edema (49). Interestingly, in all three TPA-caused skin edema protocols, the application of silymarin at a 6-mg dose 30 min prior to that of each TPA treatment in different protocols resulted in either complete (in case of single and two TPA application protocols) or 94% (in case of three TPA application protocol) inhibition (P < 0.001, Student’s t test) of TPA-caused skin edema (Table 2). Dorsal application of silymarin alone did not result in any skin edema in SENCAR mice (Table 2).

Next, we assessed the effect of preaplication of silymarin on TPA-caused induction of epidermal hyperplasia using treatment protocols identical to those used for skin edema studies. Both, qualitative and quantitative analysis were performed to evaluate the inhibitory effect of silymarin on TPA-induced epidermal hyperplasia. The skin tissue sections obtained from different treatment groups were stained with H&E for qualitative evaluation and were microscopically examined for mean vertical thickness and mean number of vertical epidermal cell layers for quantitative analysis, as detailed in “Materials and Methods.” As shown in Fig. 3, in terms of H&E staining of tissue sections, compared to single (A) or double (B) applications of acetone as vehicle, a single topical application of TPA on to the shaved SENCAR mouse skin resulted in a significant induction of epidermal hyperplasia (Fig. 3D). A further, highly significant increase in epidermal hyperplasia was evident following an additional application of TPA 48 h after the first application (Fig. 3E). Compared to single and double applications, TPA applied three times at an interval of 24 h apart resulted in much pronounced induction of epidermal hyperplasia (Fig. 3F). Interestingly, no matter whether moderate (in the case of the
PROTECTION AGAINST TUMOR PROMOTION BY SILYMARIN

Fig. 2. Preventive effect of topical application of silymarin on stage-specific skin tumor promotion in DMBA-initiated SENCAR mice. As detailed in “Materials and Methods,” three different experimental protocols were used to assess the preventive effect of silymarin against stage I, stage II, and both stage I and II tumor promotion in mouse skin. The doses of DMBA, TPA, MEZ, and silymarin in different protocols were 10 nmol, 3.2 nmol, and 6 mg, respectively. Different treatments with tumor promoters without or with prior application of silymarin were continued up to the end of the experiment at 24 weeks from the start of DMBA. The body weight (g) per mouse (A), percentage of mice with tumors (B), and number of tumors per mouse (C) were plotted as a function of the number of weeks on test. The body weight gain profile data shown are mean ± SE of 20 mice per group throughout the experiment, except in the case of the DMBA + TPA + MEZ group, in which they are mean ± SE of 18 mice after 11 weeks because two animals died at week 12 of experiment. The percentage of mice with tumor data are from 20 mice per group throughout the study. The tumors per mouse data are also mean ± SE of 20 mice per group throughout the experiment except in case of DMBA + TPA + MEZ group, in which they are mean ± SE of 18 mice after 11 weeks.

one-time TPA protocol), strong (in the case of the two-times TPA protocol), or very strong (in the case of the three-times TPA protocol) epidermal hyperplasia induced by TPA, preapplication of silymarin at 6 mg dose prior to each TPA application in different protocols resulted in an exceptionally high inhibition of TPA-induced epidermal hyperplasia (Fig. 3, G–I). A visual examination of the histology of the skin tissue sections from silymarin plus TPA groups clearly show that, in each case, epidermal hyperplasia was comparable to that observed in acetone controls. Application of silymarin alone once, twice (data not shown), or three times (Fig. 3C) did not result in any noticeable induction of epidermal hyperplasia in SENCAR mice. Similar to single (Fig. 3A) or double (Fig. 3B) applications, three applications of acetone also did not result in any appreciable induction of epidermal hyperplasia in SENCAR mice (data not shown).

Corroborating the qualitative analysis of H&E-stained tissue sections, quantitative analysis for TPA-induced epidermal hyperplasia and its inhibition by preapplication of silymarin showed similar results. As shown by data in Table 2, compared to acetone-treated controls, single, double, or triple topical application of TPA on to the shaved dorsal skin of SENCAR mice resulted in a significant increase in mean vertical epidermal thickness measured as 1.5 ± 0.2, 5.5 ± 1.1, and 7.1 ± 1.2 μm, respectively, as well as mean vertical epidermal cell layers measured as 6.8 ± 2.1, 9.4 ± 0.2, and 11.5 ± 1.6, respectively. Preapplication of silymarin to that of each TPA treatment, however, resulted in a highly significant inhibition (38–92%, P < 0.01 or 0.001, Student's t test) in terms of both mean vertical epidermal thickness and mean vertical epidermal cell layers (Table 2). Compared to acetone controls, the topical application of silymarin alone did not result in an increase in either mean epidermal thickness or mean vertical epidermal cell layers (Table 2).

Inhibitory Effect of Silymarin on TPA-caused Induction of Epidermal Proliferation. To assess the inhibitory effect of silymarin on TPA-caused induction of epidermal proliferation in SENCAR mice, identical experimental protocols to those of TPA-caused skin edema and induction of epidermal hyperplasia were followed. The shaved dorsal skin of mice was left untreated or treated topically once, twice, or three times with acetone or TPA in acetone without or with prior application of silymarin. At desired times thereafter, the treated area of the skin was removed, and tissue sections were prepared and stained with anti-PCNA antibody. The quantitative analysis of the cells that bound anti-PCNA antibody was determined by counting the PCNA positive cells per 10 arbitrary selected fields under a microscope at ×40 magnification in each case in a double-blinded manner. In each case, the number of PCNA-negative cells as well as total number of cells in the field were also counted to determine the percentage of PCNA-positive cells per treatment sample, as detailed in “Materials and Methods.” Qualitative analysis of the PCNA staining was evident by visual examination of the anti-PCNA antibody-stained tissue sections under a microscope.

As shown by quantitative analysis data in Fig. 4A, compared to single topical application of acetone treated controls showing only 20% PCNA-positive cells, single topical application of TPA resulted in a significant increase in epidermal proliferation, as evidenced by a frequency of 40% PCNA-positive cells (Fig. 4A). Additional application of TPA either once or two more times did not result in an appreciable increase of the percentage of PCNA-positive cells (Fig. 4A). This observation was sur-
Table 2  Topical application of silymarin prior to TPA results in significant inhibition of TPA-caused skin edema and epidermal hyperplasia in SENCAR mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Skin edema</th>
<th>Epidermal hyperplasia</th>
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<tbody>
<tr>
<td>Controls</td>
<td>Skin punch</td>
<td>Epidermal thickness</td>
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<tr>
<td>Acetone</td>
<td>weight (mg)</td>
<td>(μm)</td>
</tr>
<tr>
<td>15.3 ± 1.1</td>
<td>0.7 ± 0.2</td>
<td>1.9 ± 0.5</td>
</tr>
<tr>
<td>Silymarin</td>
<td>15.4 ± 1.0</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>Experimental</td>
<td></td>
<td></td>
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<tr>
<td>TPA (1×)</td>
<td>22.9 ± 1.4</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>Silymarin + TPA (1×)</td>
<td>15.0 ± 1.6</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>TPA (2×)</td>
<td>25.4 ± 1.3</td>
<td>5.5 ± 1.1</td>
</tr>
<tr>
<td>Silymarin + TPA (2×)</td>
<td>14.3 ± 1.6</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>TPA (3×)</td>
<td>36.2 ± 3.9</td>
<td>7.1 ± 1.2</td>
</tr>
<tr>
<td>Silymarin + TPA (3×)</td>
<td>16.6 ± 2.3</td>
<td>1.5 ± 0.5</td>
</tr>
</tbody>
</table>

* Skin edema was determined by weighing the 1-cm-diameter punch of different treated skin as described in “Materials and Methods.” Epidermal hyperplasia represented as vertical epidermal thickness, and no. of vertical epidermal cell layers was determined by microscopic examination of different treated skin tissue sections as detailed in “Materials and Methods.”

1  Mean ± SE of three individual values; five 1-cm skin punch biopsies from one mouse were pooled and represented as one individual value.

2  Mean ± SE of three individual values; in each case, a mean of 25 vertical epidermal measurements, equidistant from each other at 2 mm in 50 mm (linear) of skin section, represents one individual value.

3  Highly significant versus TPA alone; P < 0.001.

4  Significant versus TPA alone; P < 0.01.

Fig. 3. Inhibitory effect of silymarin on TPA-caused induction of epidermal hyperplasia in SENCAR mice. Dorsal shaved skin of mice was treated with acetone, 6 mg of silymarin in acetone, 5 nmol of TPA in acetone, or 6 mg of silymarin in 0.2 ml of acetone, followed 30 min later with 5 nmol of TPA in 0.2 ml of acetone. These treatments were performed once, twice (48 h apart), or three times (24 h apart), and 24 h after the last treatment, animals were sacrificed, and the treated area of the skin was used for the studies, as described in “Materials and Methods.” Data shown are: acetone once (A); acetone two times (B); silymarin three times (C); TPA once (D); TPA two times (E); TPA three times (F); silymarin + TPA once (G); silymarin + TPA two times (H); and silymarin + TPA three times (I). In each case, representative data are shown at ×40 magnification.

/prisingly not consistent with the multiple applications of TPA-caused induction of epidermal hyperplasia shown in Fig. 3, E and F, and Table 2. In a further analysis of the quantitative PCNA staining data, interestingly, we found that there was a clear relationship between the number of TPA applications and an increase in PCNA-positive cells, although not in terms of the percentage of PCNA-positive cells. For example, compared to acetone control counting for 257 PCNA positive cells of a total of 1286 cells (20%) per 10 fields, these numbers were 443 of 1115 (40%), 670 of 1657 (40%), and 1025 of 2434 (42%) for once-, twice-, and thrice-TPA-treated skin samples, respectively (data not shown). When we assessed the effect of silymarin on TPA-caused induction of epidermal proliferation in terms of the percentage of PCNA-positive cells, as shown by data in Fig. 4A, preapplication of silymarin in addition to single or double application of TPA resulted in a significant inhibition (55% decrease,
Fig. 4. Inhibitory effect of silymarin on TPA-caused induction of epidermal proliferation in SENCAR mice, as measured by quantitative and qualitative analysis of cell populations in skin which bound anti-PCNA antibody. Under the identical protocol detailed in Fig. 3, dorsal skin was treated once, two times (48 h apart), or three times (24 h apart) with acetone, 6 mg of silymarin in acetone, 5 nmol of TPA in acetone, or 6 mg of silymarin followed 30 min later with 5 nmol of TPA in acetone, and 24 h after the last treatment, animals were sacrificed. Treated areas of the skin from different treatment protocols were removed and fixed in formalin, and sections were prepared and immunohistochemically stained with anti-PCNA antibody for both quantitative and qualitative analysis of PCNA-positive proliferating epidermal cells as detailed in "Materials and Methods." Data shown are the summary of quantitative analysis of percentage PCNA-positive cells per 10 arbitrarily selected fields at ×40 magnification in different treatment groups (A); and photomicrographs to visually demonstrate the anti-PCNA antibody staining in one time acetone (B), three times TPA (C), and three times silymarin + TPA (D) treated mouse skin samples. In each case in B–D, representative data are shown at ×40 magnification.

P < 0.01, Student’s t test) of PCNA-positive cells in epidermis. More profound inhibitory effect of silymarin in terms of reduction in PCNA-positive cells (86% decrease, P < 0.001, Student’s t test) was observed in the protocol in which TPA application was done three times at intervals of 24 h, and silymarin was applied prior to each TPA application (Fig. 4A). Treatment of silymarin alone did not result in any increase in epidermal proliferation; as in this case, the percentage of PCNA-positive cells was even less than that observed with acetone control (Fig. 4A). Similarly, double or triple application of acetone alone also did not result in an increase in PCNA-positive cells other than those observed with single acetone application (data not shown). The anti-p53 antibody used as negative control did not show any appreciable staining (Fig. 4A). The qualitative immunohistochemical localization of PCNA in proliferating cells is shown by microscopic images of skin tissue sections stained with anti-PCNA antibody (Fig. 4, B–D). As evidenced by visual examination of the black-stained cells, compared to acetone controls (Fig. 4B), a highly significant increase in the number as well as a change in the locations of cells stained with anti-PCNA antibody was observed in the skin sample from three times TPA-treated mice (Fig. 4C). A significant reduction in this effect of TPA was clearly evident when silymarin was applied prior to each TPA application (Fig. 4D).

Inhibitory Effect of Silymarin on DNA Synthesis. On the basis of our findings showing that application of silymarin on to the dorsal shaved SENCAR mouse skin prior to single or multiple TPA treatments results in a highly significant inhibition of TPA-induced epidermal proliferation in terms of reduction in PCNA-positive cells, we also assessed the effect of silymarin on DNA synthesis. These studies were performed in culture using human epidermoid carcinoma A431 cells. Following treatment of cells with varying doses of silymarin for 4 h, they were labeled with [methyl-3H]thymidine for 18 h, and cell associated radioactivity (thymidine incorporation) was determined as the measure of DNA synthesis. As shown in Fig. 5, treatment of cells with silymarin resulted in a highly significant inhibition in a dose-dependent manner of DNA synthesis in terms of reduction in [methyl-3H]thymidine incorporation. Compared to solvent control, 7.5 μM dose of silymarin showed a 24% inhibition (P < 0.05, Student’s t test) in DNA synthesis (Fig. 5). The doses of silymarin lower than 7.5 μM did not show any appreciable inhibition of DNA synthesis (data not shown). However, much stronger inhibition of DNA synthesis (61 and 82% reduction, P < 0.001, Student’s t test) was observed at 15 and 30 μM doses, respectively, of silymarin (Fig. 5). No further inhibition in DNA synthesis was observed following treatment with the doses higher than 30 μM (data not shown).

Inhibitory Effect of Silymarin on Epidermal Lipid Peroxidation. Several evidences demonstrate that skin tumor promoters-caused oxidative stress contribute largely to their skin tumor-promoting potential (50, 51). Phorbol ester TPA has been studied extensively in this regard and shown to cause oxidative stress as one of its mechanisms of tumor promotion (50–52). If not scavenged, these oxidative stress conditions ultimately lead to highly reactive oxygen species, namely, hydroxyl radical, which attacks cellular targets such as DNA, protein, and lipid-rich membranes (53). The formation of lipid peroxides via lipid peroxidation process and their inhibition in biological membranes are a useful system to assess both oxidant and antioxidant activity of endogenous as well as exogenous agents (53, 54). To evaluate whether the observed protective effects of silymarin on tumor promotion and whether inflammatory and proliferative responses are also due to its potent antioxidant activity, studies were performed to examine its effect on lipid peroxidation using epidermal microsomes prepared from SENCAR mouse epidermis. As shown in Fig. 6, in vitro addition of silymarin to incubation mixtures resulted in a highly significant inhibition in a dose-dependent manner of...
epidermal lipid peroxidation, so much so, that a 0.1-μM concentration of silymarin showed a 18% inhibition (P < 0.1, Student’s t test) of lipid peroxidation in epidermal microsomal suspension (Fig. 6). Higher concentrations of silymarin, ranging from 0.5 to 20 μM, showed as much as 40—89% inhibition (P < 0.001, Student’s t test) of epidermal lipid peroxidation (Fig. 6). Much higher concentration of silymarin than 20 μM dose did not show any additional decrease in lipid peroxidation (data not shown).

DISCUSSION

It is recognized that most human malignancies develop from the progeny of a single cell through sequential steps as a result of biochemical, physiological, and molecular alterations (41–43). Carcinogenesis in mouse skin and other animal tumor bioassay systems and, possibly, in humans is a stepwise process of at least three distinct stages: initiation, promotion, and progression (Refs. 41–43 and references therein). For more than 50 years, mouse skin has been used as a conventional model to study the mechanism of carcinogenesis and modulation of sequential steps involved in this process (41–43). In fact, this is the oldest experimental model to demonstrate the multistep process of carcinogenesis and to define the cellular, biochemical, and molecular mechanisms associated with each stage (41–43). The mouse skin carcinogenesis model, which provides a conceptual framework to study the carcinogenesis process, has also been used extensively to: (a) assess whether chemical and/or physical agents carry a carcinogenic hazard to humans and (b) evaluate the cancer-chemopreventive effects of different agents and define the mechanism involved with their protective effects (41–43). Using the skin carcinogenesis model, we found that a naturally occurring polyphenolic flavonoid antioxidant, silymarin, is an exceptionally high anti-tumor promoting agent and that this effect of silymarin is largely due to its protective effect against stage I tumor promotion.

Several studies have shown that the human diet contains various mutagens and carcinogens, along with many antimutagens and anticarcinogens (55). Other studies have shown that many mutagens and carcinogens exert their effect via the generation of oxygen and other radicals that play a major role in the causation of cancer, specifically, at the promotion stage of carcinogenesis (50, 51, 55). Dietary intake of naturally occurring antioxidants, therefore, has been suggested to be a useful strategy against the toxic effects of these mutagenic and carcinogenic agents (Refs. 12–14 and references therein). A wide range of studies have shown that several naturally occurring compounds possess significant anti-tumor-promoting activity due to their antioxidant activity (12–14). Consistent with these studies, silymarin, which is a very strong antioxidant (25–28), also showed exceptionally high anti-tumor-promoting effect in a dose-dependent manner. One of the important findings in antitumor promotion studies with silymarin was that, at 10 weeks, only 5% of animals treated with 12-ng silymarin were still tumor-free. However, 20% of mice in the 6- and 3-ng silymarin-treated groups exhibited tumor appearance against the 100% of mice with tumors in non-silymarin-treated positive control group. These data clearly demonstrate that in addition to a delay on the onset of tumor appearance and protection against tumor incidence and multiplicity, silymarin also slows down the tumor promotion during multistage carcinogenesis process.

In our experiment to dissect out the tumor promotion stage specificity of silymarin, we observed that silymarin more specifically protects against stage I tumor promotion. Whereas the topical application of silymarin 30 min prior to TPA treatment in anti-stage II tumor promotion protocol resulted in only 1 week of delay in latency period, a 5-week delay in latency period was observed in animals treated with silymarin prior to only TPA treatment (anti-stage I protocol) as well as prior to both TPA and MEZ treatment (anti-stage I and II protocol). These data strongly suggest that the observed delay in the latency period in the anti-stage I and II protocol is largely due to the protective effect of silymarin against stage I tumor promotion. The delay in the latency period on the onset of tumor appearance by silymarin observed in three different stage-specific promotion studies also correlated with the tumor incidence and multiplicity data in these protocols. Because stage I tumor promotion is referred as “conversion,” whereby the initiated cell is converted to a dormant tumor cell which proliferate to grossly visible tumors in “propagation” or stage II promotion (36–40), the observed data with silymarin showing protective effect against stage I promotion suggest that, as an initial step, silymarin inhibits the conversion of initiated cells in the dormant tumor cells. The anti-stage I-specific skin tumor-promoting effects of silymarin observed in this study were different from those reported earlier for another antioxidant preparation, a polyphenolic fraction isolated from green tea, showing equally effective protection against both stage I and stage II skin tumor promotion under identical conditions.

**Fig. 5.** Inhibitory effect of silymarin on DNA synthesis in human epidermoid carcinoma A431 cells. The cells were cultured in DMEM with 10% FCS and 1% penicillin-streptomycin and then seeded at the density of 1000 cells per well in a 96-well plate. Cells were treated with vehicle alone or 7.5, 15, or 30 μM final concentration of silymarin for 4 h and then added with [methyl-3H]thymidine. After 18 h, cells were harvested, and [methyl-3H]thymidine incorporation was determined. The data shown as percentage of control are from mean (± SE of <8%) of three independent experiments, each performed with six wells per treatment.

**Fig. 6.** Inhibitory effect of silymarin on epidermal lipid peroxidation. Epidermal microsomes were prepared from SENCAR mice skin, and microsomal suspension (2 mg protein) in 0.1 M phosphate buffer (pH 7.4), containing 0.1 mM MgCl₂, was incubated for 1 h at 37°C in the presence of ferric ions (1 mM FeCl₃) and ADP (5 mM) in a total volume of 1 ml either with vehicle alone or varying concentrations of silymarin (0 to 20 μM). The reaction was terminated with 10% trichloroacetic acid followed by 1.2 ml of 0.5% 2-thiobarbituric acid. The generation of MDA measured at 532 nm absorbance was used as marker of lipid peroxidation and calculated using a molar extinction coefficient of 1.56 × 10⁴ M⁻¹ cm⁻¹ as detailed in “Materials and Methods.” Columns, percentages of control from mean (± SE of <10%) of three independent assays, each done in triplicate.
experimental protocols (45). Because both these agents are strong anti-oxidants (25–28, 48), the observed difference in their stage-specific tumor-promoting effects could be explained based on their structure variation and number of phenolic groups. For example, compared to silymarin that possesses only three phenolic groups (32, 33), the major constituent present in green tea isolate and responsible for most of its cancer-preventive potential, (−)-epigallocatechin 3-gallate, possesses eight phenolic groups (14, 56, 57). Recent studies in our laboratory have also focused on defining the role such structure variation and number of phenolic groups in silymarin and (−)-epigallocatechin 3-gallate play in impairing signaling pathways as a plausible mechanism of their differential stage-specific anti-tumor-promoting effects. Consistent with a recent study (58), we observed that (−)-epigallocatechin 3-gallate does not inhibit ligand- or TPA-caused activation of mitogen-activated protein kinase (erk1 and erk2), whereas silymarin showed highly significant inhibition in a dose dependent manner (59). In other studies, whereas (−)-epigallocatechin 3-gallate showed a highly significant inhibition, in a dose-dependent manner, of ligand-caused activation of survival factor AKT, silymarin was not effective. Additional studies are in progress to delineate the differential effects of silymarin and (−)-epigallocatechin 3-gallate on these antiproliferative and death-associated signaling pathways as well as their involvement in stage-specific skin tumor promotion process.

In the studies assessing the effect of silymarin on the short-term markers associated with stage I of skin tumor promotion, compared to single or multiple topical applications of TPA on SENCAR mouse skin showing a highly significant induction of skin edema, epidermal hyperplasia and proliferation index in terms of PCNA staining in the epidermis, treatment of mice with silymarin prior to TPA resulted in a highly significant inhibition of TPA-caused cellular changes. In other studies, silymarin also showed strong inhibition of DNA synthesis and lipid peroxidation which could be associated with its antiproliferative and anti-inflammatory effects at initial stage in tumor promotion studies. Furthermore, because edema formation and epidermal hyperplasia along with an increase in the number of dark basal keratinocytes, neutrophil infiltration, and lipoygenase and cyclooxygenase dependent metabolism of arachidonic acid (41–43, 60, 61) are characteristic features of stage I tumor promotion, the observed effect of silymarin on stage I tumor promotion is consistent with the findings showing its significant inhibitory effect on TPA-caused edema and induction of epidermal hyperplasia and proliferation index. In addition, the observed effect of silymarin in inhibiting stage II tumor promotion either alone or in conjunction with stage I tumor promotion, although not as remarkable as stage I tumor promotion alone, could largely be due to its inhibitory effects on tumor promoter-induced epidermal ODC activity and mRNA expression reported earlier by us (32).

Other than conventional cellular and biochemical changes following the treatment of mouse skin with TPA (38–43, 60, 61), major focus of research in recent years has been to define the role and involvement of several inflammatory cytokines (e.g., interleukin 1α and TNF-α, Refs. 35 and 62), growth factors (e.g., transforming growth factor α and β), epidermal growth factor, and insulin growth factor; Refs. 63–70), and several inflammatory cytokines (e.g., transforming growth factor α and β, epidermal growth factor receptor, transforming growth factor β receptor, and insulin growth factor receptor; Refs. 63–70) in multistage mouse skin carcinogenesis process. Several studies have shown the involvement of cytokines in TPA-caused inflammation and tumor promotion (35, 62, 71). The involvement of growth factors and their receptors has also been established in DMBA-TPA-induced papillomas and carcinomas (63–70). These studies have also shown an increase in the expression of growth factors and their receptors in mouse skin following topical application of both phorbol and non-phorbol ester skin tumor promoters establishing the involvement of receptor and nonreceptor tyrosine kinase signaling pathways in mouse skin tumor promotion (63–70).

Consistent with these molecular changes associated with tumor promotion, we have demonstrated that treatment of mouse skin with silymarin prior to TPA or okadaic acid results in a highly significant complete inhibition of TNF-α mRNA expression in a dose-dependent manner (34). In other studies, we also found that treatment of human epidermoid carcinoma A431 cells with silymarin results in almost complete inhibition of ligand-induced activation of epidermal growth factor receptor and impairment of downstream signaling pathway involving adapter protein SHC activation (72). This effect of silymarin also resulted in perturbations in cell cycle progression and growth arrest, which was largely due to an induction of cyclo-dependent kinase inhibitors Cip1/p21 and Kip1/p27 and a decrease in the kinase activity of cyclin-dependent kinases and associated cyclins in A431 cells (59, 72). It is important to mention here that, in recent years, the cell cycle regulatory molecules such as cyclin-dependent kinase inhibitors, cyclin-dependent kinases, and cyclins have been shown to be associated with both chemical- and UV radiation-induced tumorigenesis in mouse skin (73, 74). Taken together, on the basis of these studies, it can be suggested that exceptionally high anti-tumor-promoting potential of silymarin is mediated via impairment of receptor and nonreceptor tyrosine kinase signaling pathways and associated changes in cell cycle progression as one of the molecular mechanisms of the effect of silymarin. More detailed studies with silymarin using mouse skin two-stage DMBA-TPA initiation-promotion protocol, are in progression to further support this suggestion under in vivo conditions.

REFERENCES


4 R. Agarwal and N. Bhatia, unpublished results.
A Flavonoid Antioxidant, Silymarin, Affords Exceptionally High Protection against Tumor Promotion in the SENCAR Mouse Skin Tumorigenesis Model


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