BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **239,** 334–339 (1997)
ARTICLE NO. RC977375

Novel Cancer Chemopreventive Effects of a Flavonoid Antioxidant Silymarin: Inhibition of mRNA Expression of an Endogenous Tumor Promoter TNFα

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Received August 11, 1997

In this study we describe exceptionally high protective effects of silymarin, a flavonoid antioxidant isolated from milk thistle, against 12-O-tetradecanoylphorbol 13-acetate (TPA)- and okadaic acid (OA)-caused tumor promotion in SENCAR mouse skin. Pre-application of silymarin to that of TPA in 7,12-dimethylbenz(a)anthracene (DMBA)-initiated mouse skin resulted in almost complete protection in terms of tumor incidence (85%) as well as multiplicity (94%). In OA-caused tumor promotion studies, application of silymarin prior to that of OA in DMBA-initiated mouse skin resulted in a complete protection against tumorigenicity. We next assessed the effect of silymarin on TPA- and OA-caused induction of mRNA expression of tumor necrosis factor α (TNFα) which is an endogenous tumor promoter and a central mediator of tumor promotion *in vivo* in the case of both TPA and OA tumor promotion. Topical application of silymarin on mouse skin prior to that of TPA or OA resulted in a highly significant to complete inhibition in a dose-dependent manner against both TPA- and OA-caused induction of TNFα mRNA expression in mouse epidermis. These results indicate that silymarin exerts novel chemopreventive effects against tumorigenicity by inhibiting endogenous tumor promoter TNFα. Additional studies are warranted in other tumor models to further evaluate the cancer chemopreventive effect of silymarin and to define the involvement of TNFα as a molecular target for such an effect.

One practical and translational approach for cancer control is to identify and define mechanism based cancer chemopreventive agent(s) for target organs. Epidemiologic and experimental studies suggesting that consumption of fresh fruits and yellow-green vegetables reduces the human cancer incidence and mortality due to stomach, colon, breast, esophagus, lung, bladder and prostate cancers, signify the usefulness of "non nutritive minor dietary constituents" as cancer chemopreventive agents (1-6). Many new classes of chemicals, therefore, are being evaluated in animal tumor models followed by clinical trials depending on their efficacy (1-6). The involvement of oxidative stress in cancer induction and its subsequent development, and associated molecular mechanisms is becoming increasingly clear (6-8). Efforts, therefore, are being made to identify naturally occurring antioxidants which could prevent, slow and/or reverse the cancer induction and its subsequent development (1-6).

One such naturally occurring polyphenolic flavonoid antioxidant is silymarin isolated from milk thistle (*Silybum marianum* (L.) Gaertn) (artichoke is one of the members in this family) (9). For over twenty years, silymarin is used clinically in Europe for the treatment of alcoholic liver diseases (10). Silymarin is non-toxic, and there is no known LD_{50} for silymarin (11). It is a strong antioxidant capable of scavenging free-radicals (12), and several short-term studies have suggested that silymarin may be a potent anticarcinogenic agent (13 and references therein). Previously, we showed that silymarin significantly inhibits tumor promoter-caused induction of ornithine decarboxylase activity and mRNA expression in mouse epidermis (13). Recently, we also showed that silymarin exerts exceptionally high protective effects against photocarcinogenesis (14).

Cancers of the skin, lung, colon, breast, prostate, cervix, bladder, esophagus, etc arise in epithelial tissues and acquire the ability to grow as squamous cell carcinomas and invade through the basement membrane (15). The multistage model of carcinogenesis in mouse skin, therefore, has provided a conceptual framework to study carcinogenesis process and define the associated mechanisms in the tissues of epithelial origin as well as identify cancer chemopreventive agents for epithelial cancers (16). To evaluate

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0006-291X/97 $25.00

the chemopreventive effects of silymarin against the cancers of epithelial cancers (16). To evaluate the chemopreventive effects of silymarin against the cancers of epithelial origin, we assessed its anti-tumor promoting potential in mouse skin model where promotion is achieved by two different classes of chemicals namely 12-O-tetradecanoylphorbol-13-acetate (TPA) and okadaic acid (OA). Furthermore, to identify a central molecular target that is modulated as a mechanism of action of silymarin against tumor promotion, we also assessed the inhibitory effect of silymarin on these two tumor promoter-caused induction of tumor necrosis factor α (TNFα) mRNA expression. TNFα, one of the inflammatory cytokines, has shown to act as an endogenous tumor promoter and a central mediator of tumor promotion, and induces similar biochemical and biological responses as known tumor promoters (17 and references therein). So much so, the studies with both TPA and OA type tumor promoters have engendered a new tumor promotion pathway mediated by TNFα that is also assumed to play a pivotal role in the process of human carcinogenesis (17).

MATERIALS AND METHODS

Chemicals. TPA and 7,12-dimethylbenz(a)anthracene (DMBA) were purchased from Sigma Chemical Co (St. Louis, MO), silymarin from Aldrich Chemical Co (Milwaukee, WI), and OA from LC Labs (Woburn, MA). The chemicals used for RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR) were of molecular biology grade and obtained through different vendors. All other chemicals and reagents were obtained in the purest from commercially available.

Animals and tumor promotion studies. Six weeks old female SENCAR mice were purchased from Harlan-Sprague-Dawley (Indianapolis, IN). The mice were housed five per cage at 24 $\pm$ 2° C and 50 $\pm$ 10% relative humidity, subjected to a 12-h light/12-h dark cycle, and fed a Purina chow diet and water *ad libitum*. The dorsal skin was shaved using electric clippers, and mice with hair cycle in resting phase were used in all studies. The mice were randomly divided into six groups of twenty animals each. The mice in Group I were left untreated and served as negative control for any spontaneous tumor induction. To assess whether silymarin alone produces tumor promoting effects, the mice in Group II were applied topically on the dorsal shaved skin with a single 10 μg dose of DMBA in 0.2 ml of acetone per mouse as tumor initiator, and one week later treated with 6 mg dose of silymarin in 0.2 ml of acetone per mouse per application twice a week up to the end of the experiment. Mice in Groups III and IV were applied topically on the dorsal shaved skin with a single 2.5 μg dose of DMBA in 0.2 ml of acetone per mouse as tumor initiator. One week later, animals in Group III were treated topically with 0.2 ml of acetone, and in Group IV with 6 mg dose of silymarin in 0.2 ml of acetone per mouse per application. Thirty minutes following these treatments, animals in both Groups III and IV were applied topically with 2 μg dose of TPA in 0.2 ml of acetone per mouse per application. Similar to DMBA-TPA protocol, mice in Groups V and VI were applied topically on the dorsal shaved skin with a single 100 μg dose of DMBA in 0.2 ml of acetone per mouse as tumor initiator. One week later, animals in Group V were treated topically with 0.2 ml of acetone, and in Group VI with 6 mg dose of silymarin in 0.2 ml of acetone per mouse per application. Thirty minutes following these treatments, animals in both Groups V and VI were applied topically with 10 μg dose of OA in 0.2 ml of acetone per mouse per application; these doses of DMBA and OA were based on published study showing tumor promoting effect of OA in DMBA-initiated CD-1 mouse skin (17). The pre-treatment 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 ornithine decarboxylase activity (13). The tumor promoter alone (Groups III and V) or silymarin plus tumor promoter treatments (Groups IV and VI) were repeated two times per week up to the termination of the experiment at 20 weeks from the start of DMBA. Animals in all 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 greater than 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 at the termination of the experiment, the tumor volume on the back of each mouse was also computed. The statistical significance of difference between the tumor incidence in silymarin-treated and untreated groups was determined by two tailed Fisher's Exact test using StatXact version 3 program (Cytel Software Corporation, Cambridge, MA). For tumor multiplicity and tumor volume/mouse, two sample Wilcoxon rank sum test was employed. An advantage of the Wilcoxon Rank Sum test is that its validity does not depend on any assumption about the shape of the distribution of tumor multiplicities.

TNFα mRNA expression studies. All treatments were done on the 3 cm^2 shaved dorsal skin area of SENCAR mice. Control animals received a single topical application of 0.2 ml acetone only. In experimental groups, the animals were treated with a single topical application of TPA or OA at 10 μg dose in 0.2 ml acetone, and sacrificed at 6, 16, 24 and 48 hrs later. To assess the inhibitory effect of silymarin on TNFα mRNA expression in mouse skin, animals in different groups were treated with acetone alone or 2, 4, 6 and 8 mg silymarin in 0.2 ml acetone and 30 min later with TPA or OA at 10 μg dose in 0.2 ml acetone. The animals in these groups were sacrificed 6 hrs after TPA or OA application. In a separate group, mice were also treated with 6 mg silymarin in 0.2 ml acetone and sacrificed after 6 hrs. Following animal killing, the dorsal treated skin was removed and made free of connective tissue, fat and dermis. Total RNA from the epidermis was isolated by CsCl gradient as described earlier (13). In brief, epidermis was homogenized in lysis buffer (4 M guanidine isothiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl and 0.1 M 2-mercaptoethanol), and homogenate was layered on a 6 M CsCl (containing 0.1 M EDTA) cushion followed by centrifugation at 100,000 $\times$g for 24 h at room temperature. The RNA pellets were resuspended in 0.1% DEPC treated double distilled water, and RNA concentration, purity and integrity determined as previously described (13).

The TNFα mRNA expression in mouse skin following TPA or OA treatment and its inhibition by pre-application of silymarin on mouse skin was determined by RT-PCR. Briefly, in a final reaction volume of 20 μl, 1 μg of total RNA was subjected to RT with murine leukemia virus reverse transcriptase at 42°C for 30 min using a GeneAmp RNA PCR kit (Roche Molecular System, Inc. Branchburg, NJ) and following step-by-step protocol of the vendor. The cDNA thus obtained was PCR amplified using specific oligonucleotide primers for mouse TNFα and mouse house-keeping hypoxanthine-guanine phosphoribosyl transferase (HPRT) genes. The primer pair for mouse TNFα (expected PCR product size 354 bp) was obtained commercially from Clontech (Palo Alto, CA). The primer sequences for mouse HPRT were synthesized based on known gene sequence, and were forward 5′-GTTGAATACAGGCCAGACTTTGTTG-3′ and reverse 5′-GATTCAACTTGCGCTCATCTTAGGC-3′ (expected PCR product size 179 bp). The cDNA (20 μl) was amplified in a 100 μl GeneAmp PCR reaction mixture (RNA PCR kit, Roche Molecular System, Inc. Branchburg, NJ) containing 0.2 μM final concentration of each primer pair for TNFα or HPRT and 2.5 U of Taq DNA polymerase.

PCR was performed using a GeneAmp PCR System 9600 (Perkin-Elmer, Norwalk, CT). Thirty five cycles of PCR were conducted for both TNFα and HPRT, and each cycle consisted of denaturation at 94°C for 45 seconds, annealing at 60°C for 45 seconds and extension at 72°C for 2 min. An additional cycle was performed thereafter for completion of reaction which consisted of denaturation at 94°C for 45 seconds, annealing at 60°C for 45 seconds and extension at 72°C for 10 min. The PCR products were subjected to 1.5% agarose gel electrophoresis in 0.5× Tris-boric acid-EDTA buffer followed by ethidium bromide staining and photography.

RESULTS AND DISCUSSION

Topical application of silymarin prior to each TPA or OA treatment resulted in an exceptionally high protection against tumor promotion in DMBA-initiated SENCAR mouse skin. In terms of any toxic effects of topical application of silymarin, as monitored by weight gain profile, no noticeable difference was observed between silymarin treated and non-treated groups of animals throughout the experiment (data not shown). In addition, topical application of silymarin alone twice weekly on DMBA-initiated mice did not result in any tumorigenicity (data not shown). Together, these observations suggest that topical application of silymarin at 6 mg dose applied twice weekly is devoid of any apparent toxicity as well as tumor promoting effect during the entire period of experiment.

In terms of anti-tumor promotion results, when the data were analyzed for the preventive effects of silymarin on TPA-caused tumor promotion, as shown in Fig. 1 (top panel), topical application of silymarin prior to that of TPA in DMBA-initiated SENCAR mouse skin resulted in a significant reduction in tumor incidence throughout the experiment. Compared to non-silymarin treated positive group of mice, the time of appearance of first tumor was delayed by three weeks in silymarin-treated animals. At this point (14 weeks), as shown in Fig. 1 (top panel), compared to 75% mice with skin tumors in non-silymarin treated group, only 5% animals in silymarin treated group developed tumors ($p < 0.0001$). However, 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 15% of the animals in silymarin treated group exhibited skin neoplasms accounting for 85% protection ($p<0.0001$) in tumor incidence (Fig. 1, top panel). Similarly, when the tumor data were evaluated in this protocol 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, pre-application of silymarin produced highly significant protection against TPA-caused tumor promotion in mouse skin (Fig. 1, bottom panel). At the end of the experiment at 20 weeks, compared to 3.6 tumors per mouse in non-silymarin treated group, only 0.2 tumor per mouse was observed in 6 mg of silymarin-treated group accounting for 94% protection ($p<0.0001$) in tumor multiplicity (Fig. 2, bottom panel). Silymarin pre-treatment in this protocol also showed a significant reduction in total tumor volume as well as tumor volume per tumor (data not shown).

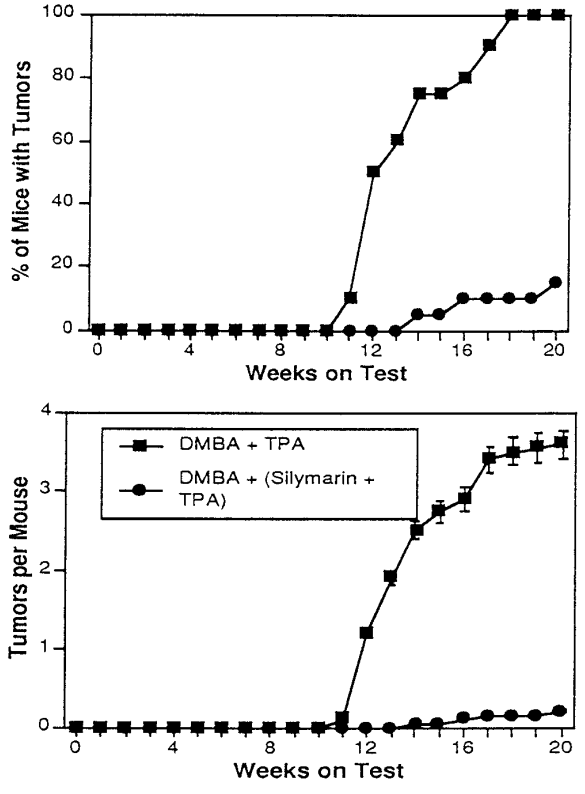

FIG. 1. Protective effect of silymarin against tumor incidence and tumor multiplicity during TPA-caused tumor promotion in DMBA-initiated SENCAR mouse skin. A single dose (2.5 μg) of DMBA was used as initiating agent, and following one week the mice were treated topically with either acetone alone or silymarin (6 mg) and 30 min later with TPA (2 μg). These treatments were performed twice weekly up to the end of the experiment at 20 weeks. Each agent was applied in 0.2 ml of acetone per application. The percentage of mice with tumors (top panel) and number of tumors per mouse (bottom panel) were plotted as a function of number of weeks on test.

In okadaic acid-caused tumor promotion protocol, topical application of silymarin at the dose of 6 mg per mouse per application prior to that of each okadaic acid application, afforded complete protection against OA-caused tumor promotion in DMBA-initiated SENCAR mouse skin (Fig. 2). The complete protective effect of silymarin was evident in terms of lack of any tumor appearance throughout the treatment protocol up to the end of experiment at twenty weeks. (Fig. 2). Since at this time point, the tumor body burden was very high in DMBA + OA positive group, we decided to terminate the study at 20 weeks. We realize that based on these results, a dose-dependent study will be needed in future to further define the protective effect of silymarin against TPA- and OA-caused tumor promotion in mouse skin.

In a standard mouse skin initiation-promotion proto-

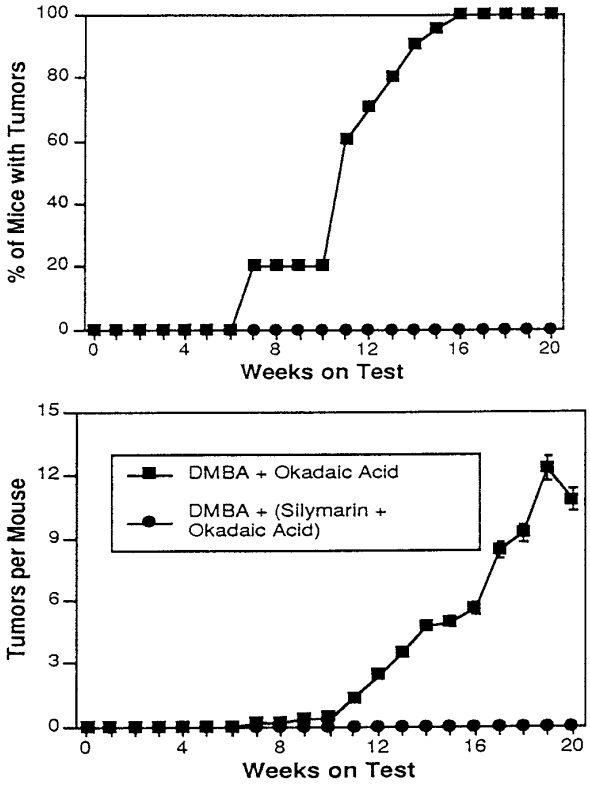

FIG. 2. Protective effect of silymarin against tumor incidence and tumor multiplicity during okadaic acid-caused tumor promotion in DMBA-initiated SENCAR mouse skin. A single dose (100 μg) of DMBA was used as initiating agent, and following one week the mice were treated topically with either acetone alone or silymarin (6 mg) and 30 min later with okadaic acid (10 μg). These treatments were performed twice weekly up to the end of the experiment at 20 weeks. Each agent was applied in 0.2 ml of acetone per application. The percentage of mice with tumors (top panel) and number of tumors per mouse (bottom panel) were plotted as a function of number of weeks on test.

col, following tumor initiation with DMBA, TPA is the most widely employed tumor promoter which activates protein kinase C (16,17). Several other skin tumor promoters, e.g. OA, are as potent as TPA in promoting skin tumors, but differ in their action from TPA as neither they compete with [^{3}H]TPA binding to a mouse skin particulate fraction nor activate protein kinase C (16,17). Therefore, there are two classes of skin tumor promoters, one which activates protein kinase C {phorbol ester (TPA) type} and other which do not {nonphorbol ester (OA) type}. A part from this basic difference in their mode of action, there are several common mechanisms too; one of them is induction of TNFα (17). In limited studies in recent years, it has been shown that both OA and TPA induce TNFα. For example, studies from Fujiki's lab showed that treatment of BALB/3T3 cells in culture with OA results in an induction in TNFα mRNA expression as well as TNFα release in culture medium, and that pre-treatment of cultures with known inhibitors of tumor promotion leads to inhibition of both OA-caused induction of TNFα mRNA expression and its release (18,19). In fact, based on these findings, the authors suggested that inhibition of TNFα mRNA expression and its release is a new process of cancer prevention (18,19). In other studies, Robertson et al (20) showed that topical application of TPA on to the mouse skin results in an induction of TNFα mRNA in the skin. However, in this study the authors showed this result at 24 hrs time point after TPA application (20) whereas in OA study the induction of TNFα mRNA expression was shown after 8 hrs of treatment (19). It is not clear from these results whether other time points were also studied. We, therefore, first performed a time-depend study to determine the induction of TNFα mRNA in mouse epidermis following TPA or OA treatment. As shown in Fig. 3A, topical application of TPA or OA on to the dorsal shaved skin of SENCAR mice resulted in a highly significant induction of TNFα mRNA expression in epidermis. Compared to acetone treated negative control, TPA treatment showed a maximum increase in this cytokine's message expression at 6 hrs followed by 16 and 24 hrs, and by 48 hrs after treatment returning to basal level (Fig. 3A). Similarly, treatment with OA also showed an increase in TNFα mRNA expression though the time-kinetics was little different from that of TPA. In this case, the expression started increasing at 6 hrs, peaked at 16 hrs and then started going back to basal level (Fig. 3A). To the best of our knowledge, this is the first study showing a time-kinetics for the increase in TNFα mRNA expression by both TPA and OA in mouse epidermis.

Based on the results shown in Fig. 3A, we selected a common 6 hrs time point to assess the inhibitory effects of silymarin against TPA- and OA-caused induction of TNFα mRNA expression. In this study, a dose-dependent effect of silymarin was evaluated to seek a co-relation between the results obtained here with those of long-term tumor promotion data. As shown in Fig. 3B, consistent with results in Fig. 3A, compared to acetone treated controls, topical application of TPA or OA on to the mouse skin resulted in a marked induction of TNFα mRNA expression in mouse epidermis. However, treatment of mouse skin 30 min prior to that of TPA or OA resulted in a highly significant inhibition in a dose-dependent manner against both the tumor promoters-caused induction of TNFα mRNA expression in mouse epidermis (Fig. 3B). In case of TPA studies, the significant inhibitory effect of silymarin was evident at as low as 2 mg dose, and at 4 mg dose a very faint band was observed. Comparable to negative control, 6 and 8 mg doses of silymarin did not show any detectable expression thus accounting for complete inhibition of TPA-caused induction of TNFα mRNA expression in mouse epidermis (Fig. 3B). Similar dose-dependent inhibitory effects of silymarin were also ob-

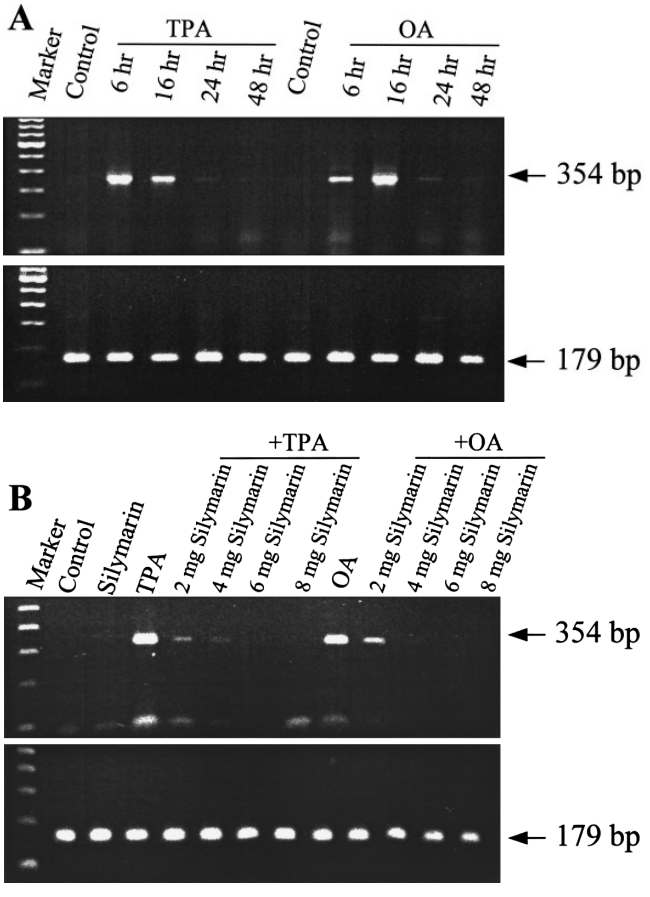

FIG. 3. Inhibitory effect of silymarin on TPA- and OA-caused induction of TNFα mRNA expression in SENCAR mouse epidermis. As detailed in Materials and Methods, shaved dorsal skin area of SENCAR mice was treated with acetone alone; a single topical application of TPA or OA at 10 μg dose in acetone for 6, 16, 24 or 48 hrs; 2, 4, 6, and 8 mg silymarin in acetone and 30 min later with TPA or OA at 10 μg dose in acetone for 6 hrs; or 6 mg silymarin in acetone for 6 hrs. Following animal killing, the dorsal treated skin was removed, total RNA from epidermis isolated, and RT-PCR performed as detailed in Materials and Methods. (A) Time-kinetics of TPA- and OA-caused induction of TNFα mRNA expression in SENCAR mouse epidermis. (B) Dose-dependent inhibitory effects of silymarin on TPA- and OA-caused induction of TNFα mRNA expression in SENCAR mouse epidermis. The 354 bp PCR product (upper panels in A and B) denotes TNFα, and the 179 bp PCR product (lower panels in A and B) shows HPRT.

served against OA-caused induction of TNFα mRNA expression in mouse epidermis but complete inhibitory effect was evident even at 4 mg dose (Fig. 3B). Treatment of mice with 6 mg dose of silymarin alone topically for 6 hrs did not result in any induction of TNFα mRNA expression (Fig. 3B). In all the RT-PCR studies for TNFα mRNA expression, HPRT was used as a housekeeping gene. As shown in the bottom panels of Fig. 3A and 3B, different tumor promoter and/or silymarin treatments did not alter the mRNA expression of HPRT. Comparing the TNFα mRNA expression results with anti-tumor promoting effects, it can be suggested that the exceptionally high and complete protective effects of silymarin at 6 mg dose against respectively TPA- and OA-caused skin tumor promotion may largely be due to complete inhibition in the tumor promoters-caused induction of TNFα mRNA expression by the same dose of silymarin. This suggestion is supported by the studies showing that TNFα is an endogenous tumor promoter and a central mediator of tumor promotion *in vivo* by both TPA and OA (17-19).

Defining the usefulness of results obtained in cancer chemoprevention studies in animal tumor models to that for human population has now become one important objective of experimental cancer research. The potential for inhibiting tumor development in targeted high-risk population in particular and in general population as a whole has increased significantly in recent years. It is, therefore, extremely important select and utilize the animal models which best represent human cancer situation(s). The validity of the multistage model of carcinogenesis in mouse skin is supported by its remarkable similarity to the changes demonstrated in human skin following chronic sun exposure leading to non melanoma skin cancers (14-16). In addition, Vogelstein and colleagues have used human colorectal carcinoma as a model system to provide convincing evidence of multistage carcinogenesis at the molecular level in a human cancer (21). In this context, the results obtained in the present study showing that a naturally occurring polyphenolic antioxidant silymarin exerts exceptionally high protective effects against tumor promotion in mouse skin carcinogenesis model, suggest that silymarin could be a useful cancer preventive agent against human cancers of epithelial origin. However, additional studies are warranted in other tumor models to further establish the usefulness of silymarin as a cancer chemopreventive agent.

ACKNOWLEDGMENT

This work was supported by United States Public Health Service Grant CA 64514 (to R.A.).

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