SHORT COMMUNICATION

Inhibitory effect of a flavonoid antioxidant silymarin on benzoyl peroxide-induced tumor promotion, oxidative stress and inflammatory responses in SENCAR mouse skin

Jifu Zhao¹, Moushumi Lahiri-Chatterjee², Yogesh Sharma¹ and Rajesh Agarwal¹,³,⁴

¹Center for Cancer Causation and Prevention, AMC Cancer Research Center, Denver, CO 80214, ²Department of Dermatology, Case Western Reserve University, Cleveland, OH 44106 and ³University of Colorado Cancer Center, University of Colorado Health Sciences Center, Denver, CO 80262, USA

Email: agarwalr@amc.org

In this communication, we investigate the preventive effect of a flavonoid antioxidant, silymarin, on free radical-generating skin tumor promoting agent benzoyl peroxide (BPO)-induced tumor promotion, oxidative stress and inflammatory responses in SENCAR mouse skin. Topical application of silymarin at a dose of 6 mg prior to BPO resulted in a highly significant protection against BPO-induced tumor promotion in 7,12-dimethylbenz[a]anthracene-initiated SENCAR mouse skin. The preventive effect of silymarin was evident in terms of a 70% reduction ($P < 0.001$) in tumor incidence, a 67% reduction ($P < 0.001$) in tumor multiplicity and a 44% decrease ($P < 0.001$) in tumor volume/tumor. In oxidative stress studies, topical application of BPO resulted in 75, 87 and 61% depletion in superoxide dismutase (SOD), catalase and glutathione peroxidase (GPX) activities in mouse epidermis, respectively. These decreases in antioxidant enzyme activities were significantly ($P < 0.005–0.001$) reversed by pre-application of silymarin in a dose-dependent manner. The observed effects of silymarin were 18–66, 32–72 and 20–67% protection against BPO-induced depletion of SOD, catalase and GPX activity in mouse epidermis, respectively. Silymarin pre-treatment also resulted in a dose-dependent inhibition (35–87%, $P < 0.05–0.001$) of BPO-induced lipid peroxidation in mouse epidermis. In inflammatory response studies, silymarin showed a strong inhibition of BPO-induced skin edema (62–85% inhibition, $P < 0.001$), myeloperoxidase activity (42–100% inhibition, $P < 0.001$) and interleukin-1α protein level in epidermis (36–81% inhibition, $P < 0.001$). These results, together with our other recent studies, suggest that silymarin could be useful in preventing a wide range of carcinogen and tumor promoter-induced cancers.

In both experimental animals and possibly in humans, cancers of the skin, lung, colon, breast, prostate, cervix, bladder and esophagus arise in epithelial tissues and acquire the ability to grow and invade through the basement membrane (1). A common pre-neoplastic precursor lesion to all squamous cell carcinomas arising in these epithelia is squamous dysplasia and/or metaplasia (2–4). Accordingly, for more than 50 years, the multistage model of carcinogenesis in mouse skin has provided a conceptual framework to study the carcinogenic process in tissues of epithelial origin (5,6). Many concepts and mechanisms of carcinogenesis as well as cancer chemoprevention now being applied to other tissues in animal model systems were originally derived from the mouse skin model (5,6). It is widely recognized that carcinogenesis in mouse skin and presumably in human skin and other tissues is a multistage process comprised of initiation, promotion and progression (5,6). Using experimental carcinogenesis models, it has become well established that oxidative stress plays a causative role during carcinogenesis, specifically in tumor promotion (7–9). This is further supported by the study where Slaga et al., for the first time, showed that benzoyl peroxide (BPO), a free radical-generating compound, induces tumor promotion in carcinogen-initiated mouse skin (10). Although BPO was found to be a weak tumor promoting agent, it was alone sufficient to induce clonal expansion of 7,12-dimethylbenz[a]anthracene (DMBA)-initiated epidermal cells into visible tumors in mouse skin (10).

Consistent with the involvement of oxidative stress in cancer induction and its subsequent development (7–9), efforts are being made to identify naturally occurring antioxidants which could prevent, slow and/or reverse cancer induction and its subsequent development (11–14). Silymarin is a flavonoid antioxidant isolated from the milk thistle (Silybum marianum) and has been used clinically for almost 25 years in Europe as an anti-hepatotoxic agent for nearly every known form of liver disease, including cirrhosis, hepatitis and necrosis (15). These effects of silymarin could be explained by alterations in liver cell membrane structure, blocking the absorption of toxins into the cells, increasing the intracellular concentration of glutathione and the antioxidant potential (16–20). Silymarin is non-toxic and there is no known LD₅₀ for silymarin (21–23).

Several short-term studies have suggested that silymarin may be a potent anti-carcinogenic agent (ref. 24 and references therein). Initially, we found that silymarin affords significant inhibition of phorbol ester and other skin tumor promoter (including BPO)-induced induction of ornithine decarboxylase activity and mRNA expression in mouse epidermis (24). More recently, we also showed that silymarin exerts an exceptionally high protective effect against UVB radiation-, 12-O-tetradecanoylphorbol 13-acetate (TPA)- and okadaic acid-induced tumor promotion in mouse skin and that the anti-tumor promoting effects of silymarin are primarily targeted at stage I tumor promotion (25–27). Based on the results of these studies, and a strong antioxidant activity of silymarin (16–20), here we have performed additional studies to investigate the preventive effect of silymarin against BPO-induced (i) tumor

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Abbreviations: BPO, benzoyl peroxide; DMBA, 7,12-dimethylbenz[a]anthracene; GPX, glutathione peroxidase; IL-1α, interleukin 1α; MDA, malondialdehyde; MPO, myeloperoxidase; ROS, reactive oxygen species; SOD, superoxide dismutase; TPA, 12-O-tetradecanoylphorbol 13-acetate.
promotion, (ii) oxidative stress and (iii) inflammatory responses in mouse skin.

Six-week-old female SENCAR mice were purchased from Harlan Sprague-Dawley (Indianapolis, IN) and maintained in our animal facility under standard conditions. The dorsal skin of the mice was shaved using electric clippers and mice in the resting phase of the hair cycle were used in all studies. For the tumor studies, the mice were randomly divided into three groups of 20 animals each. The mice in group I were left untreated and served as negative controls. Mice in groups II and III were topicaly applied on the dorsal shaved skin with a single 2.5 µg dose of DMBA in 0.2 ml of acetone/mouse as tumor initiator. One week later, animals in group II were treated topically with 0.2 ml of acetone and in group III with 6 mg of silymarin in 0.2 ml of acetone/mouse/application.

Thirty minutes following these treatments, animals in both groups II and III were applied topically with 20 mg of BPO in 0.2 ml of acetone/mouse/application. The BPO alone (group II) or silymarin plus BPO treatments (group III) were repeated twice per week up to termination of the experiment at 40 weeks from the start of DMBA. We did not include a DMBA + silymarin group in this study as we have previously shown that a 12 mg topical application of silymarin twice a week to DMBA-initiated SENCAR mouse skin does not have a tumor promoting effect (27). The selection of 6 mg of silymarin in the present study was based on a recent study by us showing that this dose causes exceptionally high anti-tumor promoting effect against both TPA- and okadaic acid-induced skin tumor promotion (26). 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 >1 mm in diameter were included in the cumulative total if they persisted for 2 weeks or more. Latent periods for the onset of tumors in groups II and III 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 differences between tumor incidence in the silymarin-treated and untreated groups was determined by two-tailed Fisher’s exact test (25,27). For tumor multiplicity and tumor volume/tumor, two sample Wilcoxon rank sum and Student’s t-tests, respectively, were used (25,27).

For antioxidant enzymes and other studies, all treatments were done on a 4 cm² shaved area. The animals were divided into six groups with four mice in each group and were treated topically on the shaved area with 0.2 ml of acetone (group I) and 0 (group II), 3 (group III), 6 (group IV) or 9 mg (group V) of silymarin/mouse each in 0.2 ml of acetone followed 30 min later by 20 mg of BPO in 0.2 ml of acetone except for group I. In group VI, the mice were treated topically with 6 mg of silymarin alone in 0.2 ml of acetone. These treatments were performed once. To assess the extent of BPO-induced edema in SENCAR mouse skin and the inhibitory effect of pre-application of different doses of silymarin, 24 h after BPO treatment a dial caliper was used to measure the skin bi-fold thickness. Edema formation was expressed as net increase in skin bi-fold thickness between BPO- and non-BPO-treated groups. Immediately after this measurement, mice were killed and the dorsal treated skin was removed and used for further studies. The epidermis was separated from the whole skin and epidermal 100 000 g supernatant and microsomal fractions prepared as described earlier (28). Superoxide dismutase (SOD) activity was measured by inhibition of reduction of nitroblue tetrazolium by superoxide anions produced by potassium superoxide dissolved in dimethylsulfoxide (29). One unit of enzyme activity is defined as the amount of enzyme causing 50% inhibition of the reduction of formazan observed in the blank. Catalase activity was determined by measuring the sample-catalyzed disappearance of H₂O₂ at 240 nm (30), and expressed as U/mg protein (1 U = OD of 1 mM H₂O₂). Glutathione peroxidase (GPX) activity was measured as disappearance of NADPH at 340 nm (31), and expressed as nmol NADPH oxidized/min/mg protein. The lipid peroxidation assay was done as detailed recently (32). Myeloperoxidase (MPO) activity was determined by the method of Bradley et al. (32) as detailed earlier (33). Epidermal interleukin 1α (IL-1α) protein levels were determined as detailed recently (34) using an ELISA kit (Endogen, Woburn, MA). For statistical analysis of the data, Student’s t-test was used between the BPO and silymarin + BPO groups.

In the studies assessing the preventive effect of silymarin on BPO-induced skin tumor promotion, as shown by the data in Figure 1, topical application of silymarin prior to BPO resulted in an exceptionally high preventive effect against BPO-induced tumor promotion in SENCAR mouse skin. No significant change was observed in the body weight gain profile between silymarin- and non-silymarin-treated groups of mice throughout the experiment (data not shown), which...
further supported the previous observation that silymarin applied topically has no apparent toxicity (25–27). In terms of tumor incidence, an exceptionally high preventive effect of silymarin against tumor promotion was evident throughout the experiment (Figure 1A). When these data were analyzed at the termination of the experiment at 40 weeks, compared with the non-silymarin-treated positive controls showing 50% of mice with skin tumors, pre-application of silymarin resulted in only 15% of mice with tumors, giving 70% protection ($P < 0.001$) against tumor incidence (Figure 1A). When the preventive effects of silymarin were analyzed for delay in latency period of the onset of first tumor, compared with the positive control group, a 5 week delay in the latency period of the onset of first tumor was observed in animals treated with silymarin prior to BPO (Figure 1A). Similar to the tumor incidence data, pre-application of silymarin also showed a highly significant protection against tumor multiplicity throughout the experimental protocol (Figure 1B). At termination of the experiment at 40 weeks, compared with $2.25 \pm 0.35$ (mean ± SE of 20 mice) tumors/mouse in the positive control group, silymarin treatment resulted in only $0.75 \pm 0.15$ (mean ± SE of 20 mice) tumors/mouse, a 67% reduction ($P < 0.001$) (Figure 1B). Interestingly, the inhibition of tumor multiplicity by silymarin also correlated with its inhibitory effect on tumor volume/tumor. Compared with the BPO alone positive group, showing $72 \pm 13$ mm$^3$ tumor volume/tumor, animals in the silymarin pretreated group showed a significantly ($P < 0.001$) smaller tumor size ($41 \pm 9$ mm$^3$ tumor volume/tumor), a 44% decrease.

Several studies have shown that topical application of skin tumor promoters induces a strong oxidative stress in mouse epidermis, which includes elevated levels of superoxide anions followed by H$_2$O$_2$ and hydroxyl radicals and depletion of antioxidant enzymes (e.g. SOD, catalase and GPX) that scavenge these reactive oxygen species (ROS) (5–9,12). This effect of tumor promoting agents on mouse skin is possibly associated with several other biochemical and molecular alterations associated with inflammation and tumor promotion (5–9). Consistent with this notion are studies showing that treatment of mouse skin with ROS and free radical-generating agents such as H$_2$O$_2$ and several different organic peroxides, including BPO, is sufficient to achieve several different cellular, biochemical and molecular alterations, including tumor promotion, as observed with TPA (5–10,35,36), and that treatment of mouse skin with antioxidants protects against these oxidative conditions and tumor promotion in skin (12–14). As silymarin is also a very strong antioxidant capable of scavenging both free radicals and ROS (16–20) and shows strong protection against BPO-induced tumor promotion, we also assessed its effect on BPO-induced depletion of antioxidant enzyme activities in mouse epidermis. The results for antioxidant enzyme activities of SOD, catalase and GPX in mouse epidermis from different groups are summarized in Figure 2. Treatment of SENCAR mice with BPO for 24 h resulted in a strong depletion of antioxidant enzyme activities in epidermis. The observed reductions in enzyme activities for SOD, catalase and GPX in mouse epidermis following a single BPO application were 75; 87 and 61% of the acetone-treated negative control, respectively (Figure 2). In the studies assessing the effect of silymarin on BPO-induced depletion of antioxidant enzyme activities, it showed a highly significant dose-dependent protection (Figure 2). In the case of SOD, pre-application of silymarin at 3, 6 and 9 mg showed 18 ($P < 0.005$), 45 ($P < 0.001$) and 66% ($P < 0.001$) protection against BPO-induced depletion of SOD activity in mouse epidermis, respectively (Figure 2A). Similarly, BPO-induced depletion of epidermal catalase activity also recovered by 32 ($P < 0.001$), 57 ($P < 0.001$) and 72% ($P < 0.001$) following pre-treatment with 3, 6 and 9 mg of silymarin, respectively (Figure 2B). In the case of GPX activity, 3, 6 and 9 mg of silymarin showed 20 ($P < 0.005$), 57 ($P < 0.001$) and 67% ($P < 0.001$) protection, respectively (Figure 2C). In contrast to these inhibitory effects of silymarin on BPO-induced depletion of antioxidant enzyme activities, treatment of mouse skin with 6 mg of silymarin alone did not result in any change in the epidermal antioxidant enzyme activities examined (Figure 2).

Under oxidative conditions, the superoxide anion is the first ROS formed, which if not scavenged, ultimately leads to a highly reactive species, the hydroxyl radical, which attacks nucleic acid, protein and lipid-rich membranes causing severe cell damage leading to genetic alterations (37,38). In this regard, the most studied markers of oxidative damage are lipid...
lipid peroxidation, as measured by MDA formation. However, application of BPO resulted in strong induction of epidermal compared with the acetone-treated control, a single topical application in mouse epidermis. As shown by the data in Figure 3, and the data shown in Figure 2, we also assessed the effect of inhibition in a concentration-dependent manner (26). Based on this study to epidermal microsomal incubations inhibits lipid peroxidation the observed inhibitory effects of silymarin were dose depend- protection against BPO-induced in a dose-dependent manner at doses of 3 and 6 mg (Figure 3). The removed, total MPO content extracted and MPO activity measured. Each data bar represents the mean ± SD of four mice; eight measurements were made at different dorsal skin sites for each mouse. The down arrow indicates a percentage decrease in BPO-induced epidermal lipid peroxidation in mice.

In recent studies we have shown that in vitro addition of silymarin to epidermal microsomal incubations inhibits lipid peroxidation in a concentration-dependent manner (26). Based on this study and the data shown in Figure 2, we also assessed the effect of pre-treatment with silymarin on BPO-induced lipid peroxidation in mouse epidermis. As shown by the data in Figure 3, compared with the acetone-treated control, a single topical application of BPO resulted in strong induction of epidermal lipid peroxidation, as measured by MDA formation. However, pre-treatment of mouse skin with silymarin resulted in significant inhibition of BPO-induced epidermal lipid peroxidation in a dose-dependent manner at doses of 3 and 6 mg (Figure 3). A further increase in silymarin dose to 9 mg showed a comparable effect to that observed at 6 mg (Figure 3). The observed inhibitory effects of silymarin at 3, 6, and 9 mg were 35 ($P < 0.05$), 80 ($P < 0.001$) and 87% ($P < 0.001$), respectively (Figure 3). Silymarin alone, however, showed almost comparable epidermal lipid peroxidation to that in the acetone-treated negative control (Figure 3).

Based on the results showing that silymarin significantly inhibits BPO-induced tumor promotion and depletion of antioxidant enzyme activities, we also assessed the effect of silymarin on BPO-induced inflammatory responses in terms of skin edema, neutrophil infiltration (by measuring MPO activity) and an increase in inflammatory cytokine IL-1α in mouse epidermis. As measured by a net increase in the skin bi-fold thickness, compared with the acetone-treated negative controls, a single topical application of BPO to mouse skin resulted in a strong increase in skin bi-fold thickness (edema) in SENCAR mice (Figure 4). However, the application of different doses of silymarin prior to BPO treatment resulted in a highly significant inhibition of BPO-induced skin edema (Figure 4). The observed inhibitory effects of silymarin were dose dependent and evident as 62, 77 and 85% inhibition ($P < 0.001$) at 3, 6 and 9 mg of silymarin, respectively (Figure 4). Similar to other studies, silymarin alone applied topically at 6 mg showed no skin edema (Figure 4).

Topical application of the tumor promoter TPA to mouse skin has been shown to result in neutrophil infiltration that is responsible, at least in part, for the oxidative conditions following TPA application to mouse skin (39–42). In a given tissue, MPO is utilized as a marker to quantify the total number of neutrophils present therein (32). Based on the data showing that silymarin protects against BPO-induced depletion of antioxidant enzyme activities and BPO-induced skin edema, we next assessed its effect on BPO-induced neutrophil infiltration by measuring MPO. As shown in Figure 5, 24 h after application of a single dose of BPO to mouse skin, total MPO activity increased from not detectable to 53 U/mg epidermal protein. This increase in the level of tissue MPO indicates an influx of neutrophils into the inflamed skin. Pre-application of silymarin before BPO, however, resulted in a significant protection against BPO-induced infiltration of neutrophils in a dose-dependent manner (Figure 5). Compared with the BPO-treated samples, 3 mg of silymarin showed 42% protection ($P < 0.001$) against BPO-induced total MPO content (Figure 5). Much stronger effects of silymarin were observed at 6 and 9 mg, which showed 77% and complete inhibition ($P < 0.001$).
of BPO-induced neutrophil infiltration in skin, respectively (Figure 5). Together, these data are consistent with an inhibitory effect of silymarin on BPO-induced depletion of antioxidant enzyme activities and skin edema and suggest that protection against BPO-induced neutrophil infiltration by silymarin is possibly responsible, at least in part, for its observed inhibitory effect on BPO-induced tumor promotion.

Cytokines have been implicated in a variety of physiological and pathological conditions, including inflammation and tumor promotion (34,43). Several studies in recent years have shown the involvement of IL-1α in both phorbol and non-phorbol ester type tumor promoter (including BPO)-induced skin inflammation and tumor promotion (34,43). We performed studies to assess the effect of silymarin on BPO-induced IL-1α protein expression in mouse epidermis. As shown in Figure 6, compared with the acetone control, a single topical application of BPO resulted in strong expression (6.5-fold increase over control) of IL-1α protein in mouse epidermis. Pre-treatment of mice with silymarin, however, showed a strong inhibition of BPO-induced IL-1α protein expression in mouse epidermis. The observed inhibitory effect of silymarin was dose-dependent and evident in terms of 36 (P < 0.005), 72 (P < 0.001) and 81% (P < 0.001) decreases in BPO-induced IL-1α protein expression in mouse epidermis at 3, 6 and 9 mg, respectively (Figure 6). Treatment of mice with silymarin alone at 6 mg did not result in any change in epidermal IL-1α protein level (Figure 6).

BPO is a free radical-generating compound and a strong oxidizing agent to which humans are constantly exposed due to the fact that BPO is widely used as: (i) a polymerization initiator; (ii) a bleaching agent for flour and cheese; (iii) as an additive in cosmetics and pharmaceutical products including those for the treatment of acne (ref. 10 and references therein). As BPO has been found to cause irritation of human skin, extensive studies were performed which showed that though not a complete carcinogen, BPO is a weak tumor promoting agent (10). Since it can be argued that tumor initiation is almost inevitable, as evident by a steady increase in cancer incidence world wide (44), a prevention strategy against tumor promotion could be a more practical approach to slow down the clonal expansion of initiated cells in the first place (5,6).

In this regard, the results of the present study showing that silymarin protects against BPO-induced tumor promotion may be of great significance in preventing any tumor promotion risk associated with BPO exposure. Taken together, based on our recent studies and the results of the present study, we suggest that silymarin could be a useful cancer preventive agent against a wide range of carcinogen and tumor promoter-induced epithelial cancers in humans.

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References


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