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Silymarin inhibits UV radiation-induced immunosuppression through augmentation of interleukin-12 in mice

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Abstract

We have shown previously that silymarin, a plant flavonoid, inhibits UVB-induced photocarcinogenesis in mice. As UVB-induced immunosuppression has been implicated in the development of skin cancer, we investigated whether silymarin can modulate the effects of UVB radiation on the immune system. Treatment of C3H/HeN mice with topically applied silymarin (0.5 or 1.0 mg/cm^2) or silibinin, a major component of silymarin, markedly inhibited UVB (180 mJ/cm^2)–induced suppression of contact hypersensitivity response in a local model of immunosuppression and had a moderate inhibitory effect in a systemic model of contact hypersensitivity. Silymarin reduced the UVB-induced enhancement of the levels of the immunosuppressive cytokine, interleukin (IL)-10, in the skin and draining lymph nodes and enhanced the levels of the immunostimulatory cytokine, IL-12. Intraperitoneal injection of mice treated with silymarin with an endotoxin-free neutralizing anti-IL-12 antibody abrogated the protective effects of the silymarin against UVB-induced suppression of the contact hypersensitivity response. Furthermore, the treatment of silymarin did not prevent UVB-induced suppression of the contact hypersensitivity response in IL-12 knockout mice but prevented it in their wild-type mice. Moreover, i.p. injection of IL-12 to silymarin-treated or non-silymarin-treated IL-12 knockout mice resulted in an enhanced response to contact hypersensitivity compared with the response in mice that were exposed to either UVB alone or silymarin plus UVB. These data indicate for the first time that silymarin has the ability to protect mice from UVB-induced immunosuppression and that this protective effect is mediated, at least in part, through IL-12. [Mol Cancer Ther 2006;5(7):1660–8]

Introduction

Solar UV radiation, particularly UVB (290–320 nm) spectrum, can act as a tumor initiator (1), tumor promoter (2), and cocarcinogen (3, 4). Exposure of skin to UVB radiation results in a variety of biological effects, including inflammation, induction of oxidative stress, formation of sunburn cells, and immunologic alterations, all of which play important roles in the development of melanoma and nonmelanoma skin cancers (3–7). UVB radiation has multiple effects on the immune system (8, 9), resulting in adverse effects on human health, including exacerbation of infectious diseases and greater risk of skin cancer (10–12). It has been recognized that chronically immunosuppressed patients living in regions of intense sun exposure experience an exceptionally high rate of skin cancer, particularly in sun-exposed areas (13). In addition, an increased incidence of skin cancers, especially squamous cell carcinomas, has been noted among recipients of organ transplants (14–16). This increased incidence of squamous cell carcinoma in transplant patients is presumably attributable to long-term immunosuppressive therapy (17), although nonimmune mechanisms also may play a role (18). Thus, a considerable body of evidence implicates UV-induced immunosuppression in the development of melanoma and nonmelanoma skin cancers.

There is great interest in the use of naturally occurring botanicals for the photoprotection of the skin. Botanicals, specifically those that possess anti-inflammatory, immunomodulatory, and antioxidant properties, are among the most promising group of agents and may represent ideal photoprotective agents (19). We have shown previously that plant polyphenols, such as (−)-epigallocatechin-3-gallate from green tea, prevent UVB-induced immunosuppression in mice and have further shown that this acts, at least in part, through augmentation of the immunoregulatory cytokine, interleukin (IL)-12 (20). To determine whether other botanical polyphenolic compounds exert similar effects, we extended our studies to examine the effects of silymarin, a flavonoid that is isolated from the fruits and seeds of the milk thistle (*Silybum marianum* L. Gaertn.). Silymarin is composed primarily of silibinin (≈90%) together with small amounts of other silibinin stereoisomers, such as isosilybin, dihydrosilybin, silydianin, and silychristin (21). As silymarin has been shown to have anti-inflammatory, antioxidative, and anticarcinogenic effects against UVB radiation *in vitro* and *in vivo* animal models (2, 22), it has been tested in various *in vitro* and *in vivo* models for its efficacy in prevention of skin

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carcinogenesis (22). We have shown previously that topical application of silymarin to sensitive-to-carcinogen mice resulted in inhibition of 7,12-dimethylbenz(*a*)anthracene-initiated and 12-*O*-tetradecanoylphorbol-13-acetate-promoted skin tumorigenesis in terms of tumor incidence, tumor multiplicity, and tumor growth (23). We also have shown that topical application of silymarin inhibits photocarcinogenesis in SKH-1 hairless mice (2). Thus, the chemopreventive studies conducted in both chemical carcinogenesis and photocarcinogenesis skin models indicated that silymarin possesses anticarcinogenic effects (2, 22, 23).

As UVB-induced immunosuppression has been implicated as a risk factor for the development of melanoma and nonmelanoma skin cancers (8, 24), we investigated the effects of silymarin treatment of SKH-1 hairless mouse skin and found that it both inhibited the generation of UVB-induced markers of oxidative stress and UVB-induced increases in the immunosuppressive cytokine, IL-10 (25). We have now extended these studies in a preclinical study in which we determined whether silymarin, when given topically, prevents UVB-induced immunosuppression in mice. The UVB-induced immunosuppression was assessed *in vivo* through measurement of allergic contact hypersensitivity responses to both low-dose and high-dose models of UVB radiation-induced immunosuppression as it has been shown that the exposure of mouse skin to UVB radiation suppresses the development of allergic contact hypersensitivity, a prototypic T-cell-mediated immune response (26, 27) through both local and systemic effects (28). We further examined the mechanism by which silymarin exerts its immunoprotective effect against UVB radiation-induced immunosuppression using various approaches, including an IL-12-knockout (KO) mouse model and analysis of the balance of the immunoregulatory cytokines IL-10, which contributes in the immunosuppressive effects of UVB radiation through the inhibition of tumor antigen presentation by epidermal antigen-presenting cells (29), and IL-12, which augments cell-mediated immune responses (30, 31), at the site of UVB exposure and in the draining lymph nodes.

Materials and Methods

Animals

Pathogen-free female C3H/HeN mice (6–7 weeks old) were purchased from Charles River Laboratory (Wilmington, MA). The IL-12 KO mice on C3H/HeN background were generated after knockdown of the p35 chain of IL-12 as described previously (32). The animals were maintained and bred (IL-12 KO) in our animal resource facility under the following housing conditions: 12-hour dark/12-hour light cycle, 24 ± 2°C temperature, and 50 ± 10% relative humidity. The mice were fed a standard Purina chow diet (Harlan Teklad, Madison, WI) and water *ad libitum*. The experimental animal protocol was approved by Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

Chemicals and Reagents

Silymarin, silibinin, and 2,4-dinitrofluorobenzene (DNFB) were purchased from Sigma Chemical Co. (St. Louis, MO). The endotoxin-free monoclonal antibody to mouse IL-12 (rat IgG1, clone C15.6) and the mouse recombinant IL-12 were purchased from eBioscience (San Diego, CA). Cytoscreen US mouse IL-10 and IL-12 ELISA kits were obtained from BioSource International, Inc. (Camarillo, CA).

UVB Light Source and Irradiation of Mice

The clipper-shaved dorsal skin of the mice was exposed to UVB radiation (180 or 1,000 mJ/cm^2) from a band of four UVB lamps (Daavlin, UVA/UVB Research Irradiation Unit, Bryan, OH) equipped with an electronic controller to regulate UVB dosage at the fixed distance of 24 cm from the lamps to the dorsal skin surface of the mice. Short wavelengths of UV (<290 nm), which are not present normally in natural solar light, were filtered out using Kodacel cellulose film (Eastman Kodak Co., Rochester, NY). The majority of the resulting wavelengths were in the UVB (290–320 nm; ~80%) and UVA (~20%) range, with peak emission at 314 nm as monitored regularly. During UVB irradiation, the ears of mice were protected from UVB using opaque black tape, which was removed after exposure. Mice that were not exposed to UVB radiation also were shaved to enable informative comparison.

UVB-Induced Local and Systemic Immunosuppression Models

The shaved dorsal skin was exposed to UVB radiation (180mJ/cm^2) to induce immunosuppression in mice. Three days later, the mice were treated topically with 25 μL of 0.5% DNFB in acetone/olive oil (4:1, v/v) either at the UVB-irradiated site (local suppression of contact hypersensitivity) or at a shaved non-UVB-irradiated ventral or distant site (systemic suppression of contact hypersensitivity). The contact hypersensitivity response was elicited 5 days later by challenging both surfaces of the ears of each mouse with 20 μL of 0.2% DNFB in acetone/olive oil (4:1, v/v). The ear thickness was measured 24 hours after the challenge using an engineer's micrometer (Mitutoyo, Tokyo, Japan) and was compared with the ear thickness just before the challenge. Mice that received the same treatment with DNFB but were not UVB irradiated served as a positive control. Non-UVB-irradiated mice that received only ear challenge without sensitization with DNFB served as negative controls. In all the experiments silymarin or silibinin was applied topically (1.0, 2.5, or 5 mg/200 μL acetone/5 cm^2 mouse skin) 25 to 30 minutes before UVB irradiation or just after UVB irradiation (within 5 minutes) on the UVB-irradiated dorsal skin sites. The mice belong to the negative and positive control groups were treated with the same amount of acetone (200 μL/mouse) topically on the dorsal skin to maintain the identical regimen. The percent suppression of contact hypersensitivity in each mouse was determined as detailed previously (20).

In vivo Treatment with Anti-IL-12 Monoclonal Antibody or Recombinant IL-12

To assess the effect of anti-mouse IL-12 antibody on silymarin-induced prevention of UVB-induced suppression

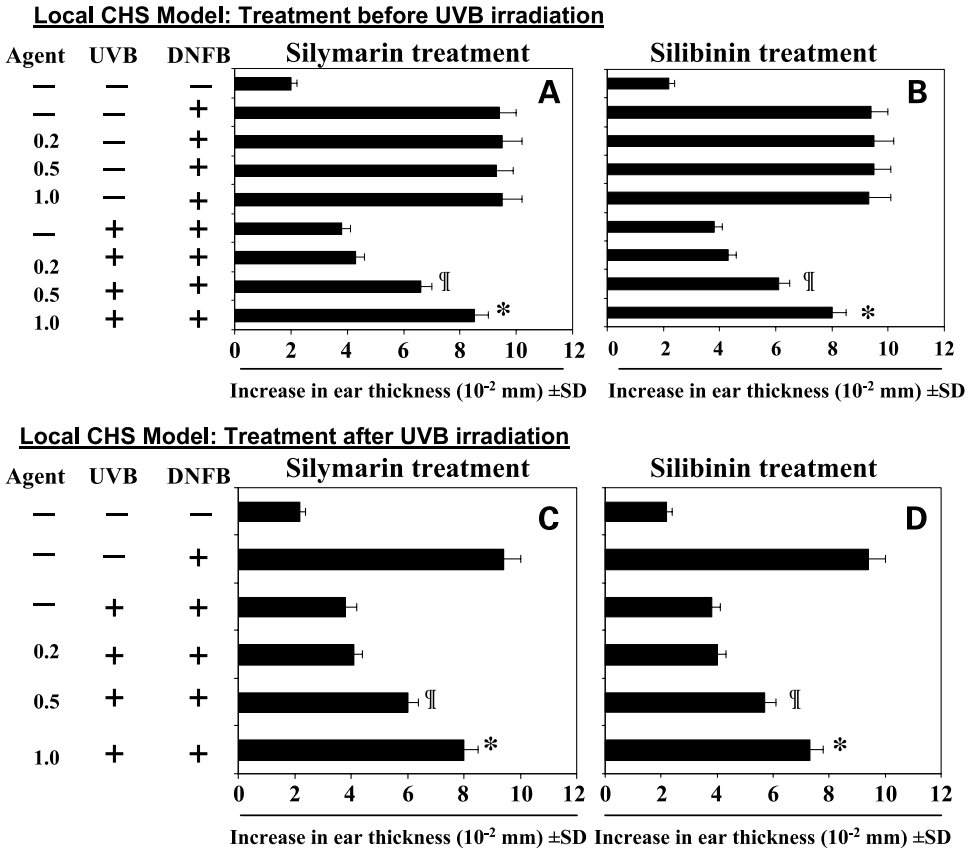

Figure 1. Silymarin and silibinin inhibit UVB-induced suppression of the contact hypersensitivity (*CHS*) response in local contact hypersensitivity model in C3H/HeN mice. The UVB-irradiated mice that did not receive treatment with silymarin or silibinin did not exhibit a significant response on DNFB challenge when sensitized through the UVB-irradiated skin (local immunosuppression). Mice that were treated with silymarin or silibinin whether before (**A** and **B**) or after (**C** and **D**) UVB exposure induce a contact hypersensitivity response in a dose-dependent manner (0.2, 0.5, or 1.0 mg/cm^2). Silymarin or silibinin treatment did not affect the ability of the mice to generate contact hypersensitivity response to DNFB (**A** and **B**, columns 3 – 5 from the top). *Columns,* mean change in ear swelling response in each group (n = 5 per group); *bars,* SD. Experiments were repeated twice with similar results. *, P < 0.001, significant versus non-silymarin-treated or non-silibinin-treated (UVB alone) animals; ¶, P < 0.005, significant versus non-silymarin-treated or non-silibinin-treated animals.

of the contact hypersensitivity response in mice, the anti-IL-12 antibody was diluted in sterile endotoxin-free saline and injected i.p. The mice received two doses of anti-IL-12 antibody (500 ng each) 24 and 3 hours before DNFB sensitization. Control mice were injected i.p. with an equal volume of rat IgG1 (isotype control of anti-IL-12) in saline, which was found to have no effect on the outcome of the sensitization procedure or on the immunosuppressive effect of UV irradiation. Recombinant murine IL-12 (1,000 ng/100 μL PBS) or an equal volume of PBS was injected i.p. 3 hours before DNFB sensitization.

Skin Homogenates for IL-10 and IL-12 Assay

In a separate set of experiment, mice were sacrificed 48 hours after UVB (180 mJ/cm^2) exposure, skin samples were collected aseptically, and s.c. tissues were removed. Skin samples obtained from non-UVB-exposed mice served as a control. The skin samples were homogenized in Tris-HCl (pH 7.5) and centrifuged at 7,000 rpm for 5 minutes. The supernatants were collected and recentrifuged at 13,000 rpm for 20 minutes, and supernatants from this centrifugation step were collected for the estimation of IL-10 and IL-12 using ELISA kits according to the manufacturer's protocol.

Preparation of Single-Cell Suspensions from Draining Lymph Nodes for IL-10 and IL-12 Assay

Single-cell suspensions from inguinal or draining lymph nodes were prepared as described previously (20). Briefly, lymph nodes were collected aseptically from different treatment groups 48 hours after UVB irradiation. The draining lymph nodes from non-UVB-exposed mice served as controls. The lymph nodes were ruptured using scissors in PBS containing 5% fetal bovine serum. The cells were filtered through 50 μm nylon mesh, resuspended in RPMI 1640 plus 10% fetal bovine serum (1×10^6 cells/0.5 mL), kept in an incubator for 24 hours, and thereafter centrifuged at 14,000 rpm for 5 minutes after which the supernatants were collected for the IL-10 and IL-12 ELISA assays.

Statistical Analysis

The statistical significance of difference in the ear swelling responses in the contact hypersensitivity experiments was done using ANOVA followed by post hoc test, whereas Student's t test was used in case of IL-10 and IL-12 levels among different treatment groups. P < 0.05 was considered significant.

Results

Both Silymarin and Silibinin Inhibit UVB-Induced Local Immunosuppression

As UVB-induced immunosuppression is considered to be a risk factor for photocarcinogenesis (8, 24) and topical treatment of silymarin prevents photocarcinogenesis in mice (2), we determined whether application of silymarin protects against UVB-induced suppression of the contact hypersensitivity response to DNFB in a model of local

UVB-induced immunosuppression in which we measure the contact hypersensitivity response to DNFB. We first confirmed that topical treatment of mouse skin with various concentrations of silymarin (0.2, 0.5, and 1.0 mg/cm^2) did not affect the ability of the mice to generate a local contact hypersensitivity response to DNFB in the absence of UVB irradiation (compare Fig. 1A, columns 3–5 from the top, with Fig. 1A, column 2 from the top, positive control). We then confirmed that in the absence of treatment with silymarin the local contact hypersensitivity response in terms of ear swelling was significantly lower (76% suppression, $P < 0.001$; Fig. 1A, column 6 from the top) in those mice that were UVB irradiated than those mice that were not UVB irradiated (Fig. 1A, column 2 from the top, positive control), indicating the immunosuppressive effect of the UVB radiation. The group of mice that were treated with silymarin, through topical administration at a concentration of either 0.5 or 1.0 mg/cm^2, before UVB irradiation exhibited a significantly lower level of UVB-induced suppression of contact hypersensitivity (61% lower, $P < 0.005$ and 87% lower, $P < 0.001$, respectively) than UV-irradiated mice that were not treated with silymarin. Topical administration of a lower concentration of silymarin (0.2 mg/cm^2) failed to provide significant protection from the UVB-induced suppression of the local contact hypersensitivity response in mice. These data indicate that the treatment doses of 0.5 or 1.0 mg/cm^2 of silymarin are capable of protecting mice from UVB-induced immunosuppression in a local model of immunosuppression.

As silibinin is the major component of silymarin (≈90%) and has been shown to have antiphotocarcinogenic effects similar to that of silymarin (33), we also assessed the effects of silibinin to determine if there was a significant difference between silymarin and silibinin in terms of their chemopreventive potential against UVB-induced suppression of contact hypersensitivity response. Using an animal protocol and experimental conditions identical to those described above for the evaluation of silymarin, we found that topical application of silibinin resulted in significant protection from UVB-induced suppression of contact hypersensitivity with 54% ($P < 0.005$) inhibition at the dose of 0.5 mg/cm^2 and 79% ($P < 0.001$) inhibition at a dose of 1.0 mg/cm^2 (Fig. 1B). Although the magnitude of protection against UVB-induced suppression of contact hypersensitivity seemed to be greater for silymarin than silibinin, this difference was not statistically significant.

Further, to exclude the possibility that silymarin or silibinin mimics a sunscreen effect, silymarin and silibinin were applied topically on UVB-exposed skin just after UVB irradiation and their effects on UVB-induced suppression of contact hypersensitivity were determined following the identical contact hypersensitivity protocol. As shown in Fig. 1C and D, topical application of silymarin or silibinin significantly inhibited UVB-induced suppression of contact hypersensitivity response to DNFB. In this case, however, the inhibition of UVB-induced suppression of contact hypersensitivity response by silymarin or silibinin was slightly less, but the difference was not significant compared with the treatment of these agents when applied before UVB irradiation (Fig. 1A and B).

Both Silymarin and Silibinin Inhibit UVB-Induced Systemic Immunosuppression

We next determined whether topical treatment of silymarin or silibinin induces inhibitory effects in a systemic model of UVB-induced immunosuppression. As in the local model of contact hypersensitivity, neither silymarin nor silibinin (0.2, 0.5, or 1.0 mg/cm^2) affected the ability of the mice to generate a systemic contact hypersensitivity response to DNFB in the absence of UVB irradiation (Fig. 2A and B, compare columns 3–5 from the top with column 2 from the top). In the systemic model

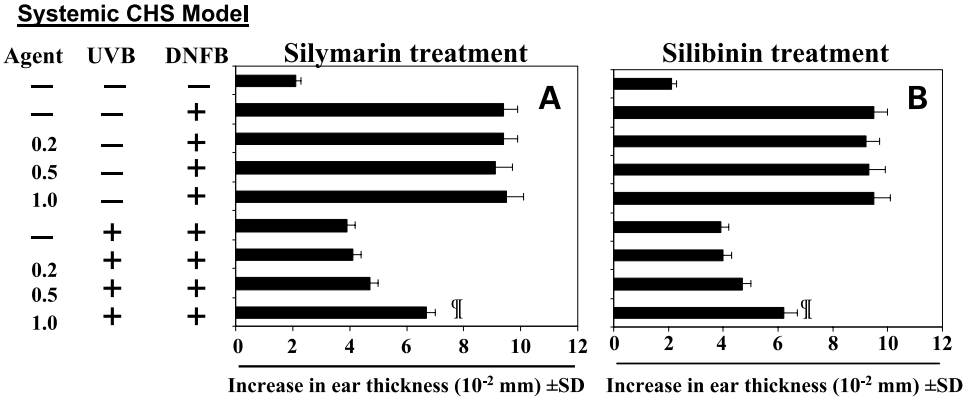

Figure 2. Silymarin and silibinin inhibit UVB-induced suppression of contact hypersensitivity response in systemic model of contact hypersensitivity in C3H/HeN mice. The UVB-irradiated mice that did not receive treatment with silymarin or silibinin did not exhibit a significant response on DNFB challenge when sensitized through the non-UVB-irradiated distant abdominal skin site (systemic immunosuppression). Mice that were treated with silymarin or silibinin before UVB irradiation on the dorsal site were able to induce a contact hypersensitivity response preferably at the dose of 0.5 and 1.0 mg/cm^2. Silymarin or silibinin treatment did not affect the ability of the mice to generate contact hypersensitivity response to DNFB (**A** and **B**, columns 3 – 5 from the top). *Columns,* mean change in ear swelling response in each group ($n = 5$ per group); *bars,* SD. Experiments were repeated twice with similar results. ¶, $P < 0.005$, significant versus non-silymarin-treated or non-silibinin-treated animals.

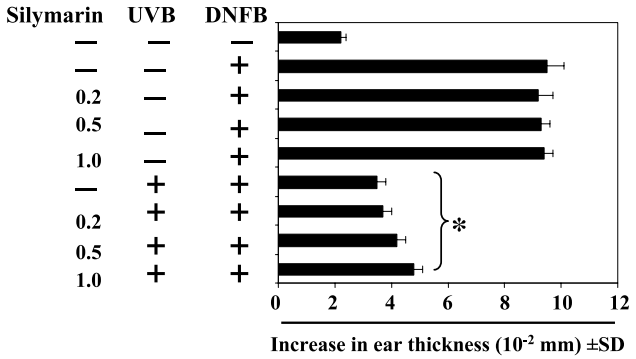

Figure 3. Silymarin does not protect against UVB-induced immunosuppression associated with an acute high-dose UVB radiation. The dose-dependent effect of silymarin (0.2, 0.5, and 1.0 mg/cm^2 skin area) on high dose of UVB irradiation (1,000 mJ/cm^2) – induced local immunosuppression was determined. Mice were treated as described in Materials and Methods. All UVB-irradiated mice exhibited a significantly lower ear swelling response to DNFB sensitization compared with the positive control group (column 2 from the top). *Columns,* mean change in ear swelling response in different treatment groups (n = 5 per group); *bars,* SD. Similar results were obtained when the experiment was repeated. *, $P < 0.001$, significant suppression of contact hypersensitivity response versus positive control.

of contact hypersensitivity, topical treatment of the lower doses of silymarin or silibinin (0.2 or 0.5 mg/cm^2) did not result in a statistically significant inhibition of UVB-induced immunosuppression compared with the positive control. Topical application at the higher dose of 1.0 mg/cm^2 skin area significantly inhibited the immunosuppressive effects of UV radiation in the systemic model of contact hypersensitivity, with silymarin inhibiting UVB-induced immunosuppression by 63% ($P < 0.005$) and silibinin inhibiting UVB-induced immunosuppression by 56% ($P < 0.005$); however, the magnitude of the immunoprotective effect in the systemic contact hypersensitivity model was lower than that observed in the local contact hypersensitivity model (Fig. 1A and B). The prevention of UVB-induced suppression of systemic contact hypersensitivity response by silymarin or silibinin may be due to the induction of immune response in animals against UVB-induced adverse effects.

Silymarin Does Not Protect against UVB-Induced Immunosuppression in a High-Dose UVB Model

To examine the effects of silymarin on the local contact hypersensitivity response to acute high-dose UVB radiation in C3H/HeN mice (Fig. 3), the shaved dorsal skin of the mice was exposed to a single 1,000 mJ/cm^2 dose of UVB, which was a 5-fold higher dose than that used in the studies described above. Otherwise, the protocol was identical to that described above. The ear swelling response was reduced significantly ($P < 0.001$) in the mice irradiated with the high dose of UVB, including those mice which were treated with silymarin at doses of 0.2, 0.5, or 1.0 mg/cm^2 (Fig. 3, columns 7–9 from the top). These results suggest that silymarin, at least under these experimental conditions, does not prevent mice from developing local immunosuppression in response to abnormally high dose of UVB irradiation.

Silymarin Reduces the UVB-Induced Increase in IL-10 Levels in the Skin and Draining Lymph Nodes

Taken together, the studies indicated that silymarin and silibinin exhibit very similar effects in terms of protection against UVB-induced immunosuppression (Figs. 1 and 2). In addition, they established that treatment of mice with silymarin or silibinin at a dose of 1 mg/cm^2 skin area conferred significant protection against UVB-induced suppression of contact hypersensitivity in both local and systemic models of contact hypersensitivity. We therefore used silymarin at a dose of 1 mg/cm^2 in a series of experiments designed to further characterize the mechanisms by which silymarin exerts its photoprotective effects.

UVB irradiation of the skin results in enhanced levels of IL-10 (20, 34), which has been implicated in UVB-induced immunosuppression (35, 36). Quantitative analysis of IL-10 using ELISA assays of skin homogenates confirmed that UVB irradiation of mice resulted in enhanced production of IL-10 in the skin (Fig. 4A). Topical application of silymarin resulted in significant inhibition 66% (n = 5; $P < 0.005$) of the UVB-induced enhancement of the levels of IL-10 in the skin when the levels of IL-10 were determined 48 hours after UVB irradiation. Treatment of silymarin did not alter the levels of IL-10 in the skin of mice that were not UVB irradiated (data not shown). As IL-10 is thought to act as an immunosuppressive cytokine, the ability of silymarin to

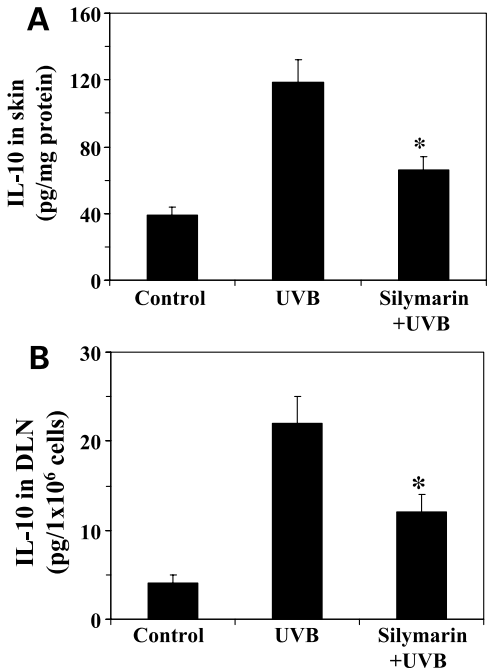

Figure 4. Silymarin inhibits the UVB-induced increase in IL-10 production in the skin and draining lymph nodes. The concentrations of IL-10 protein in skin homogenates (**A**) and that produced by the cells from lymph nodes (**B**) were determined by ELISA as detailed in Materials and Methods. *Columns,* mean amount of IL-10 protein expressed as either pg/mg protein or pg/1 × 10^6 cells (n = 5 per group); *bars,* SD. Experiments were repeated at least once with similar results. *, $P < 0.005$, significant reduction versus UVB alone control group.

prevent the UVB-induced enhancement of the levels of IL-10 in the skin may contribute to its ability to prevent UVB-induced suppression of the contact hypersensitivity response.

Using a similar approach, we determined the levels of IL-10 by the cells of the draining lymph nodes of the mice in the different treatment groups. In mice that were not UVB irradiated, only low levels of IL-10 were detected in the cells of the draining lymph nodes whether they were treated with silymarin (data not shown; Fig. 4B). UVB irradiation of mouse skin resulted in enhancement of the levels of IL-10 (~6-fold) at 48 hours after UVB irradiation by the draining lymph node cells of mice that were not treated with silymarin. Treatment with silymarin resulted in a significant reduction (56%) in the UVB-induced levels of IL-10 in the draining lymph node cells ($n = 5$; $P < 0.005$) as shown in Fig. 4B.

Silymarin Increases the Levels of IL-12 in the Skin and Draining Lymph Nodes of UVB-Irradiated Mice

IL-12 has been shown to prevent UVB-induced suppression of contact hypersensitivity response in mice (37) through stimulation of T-cell-mediated immune responses (26, 31); therefore, we examined the effect of silymarin on the induction of IL-12 in C3H/HeN mice. Quantitative analysis of IL-12 in skin homogenates by ELISA confirmed that exposure of the mice to UVB results in enhanced levels of IL-12 in the skin (76 ± 9 versus 38 ± 5 pg/mg protein for non-UVB-irradiated control mice). Treatment with silymarin significantly enhanced the levels of IL-12 in the skin of the UVB-irradiated mice (145 ± 14 pg/mg protein; $P < 0.005$) at 48 hours after UVB irradiation (Fig. 5A). We also determined the levels of IL-12 in the draining lymph node cells (Fig. 5B) using a protocol similar to that used for the quantification of IL-12 in the skin, except that the levels were calculated in terms of the quantity of IL-12 per million cells. We found that UVB irradiation of the mouse skin resulted in an ~3-fold enhancement of IL-12 in the draining lymph node cells. Treatment of UVB-irradiated mice with silymarin resulted in even greater levels of IL-12 in the lymph nodes, with the levels of IL-12 being ~2-fold higher in UVB-irradiated mice that were treated with silymarin ($P < 0.005$) than those that were not treated with silymarin (Fig. 5B). This further enhancement in the levels of IL-12 on silymarin treatment of the UVB-irradiated mice seemed to be a synergistic effect as silymarin treatment of non-UVB-exposed mouse skin did not affect the basal levels of IL-12 in the draining lymph node cells (data not shown).

Treatment of Mice with Anti-IL-12 Antibody Inhibits the Ability of Silymarin to Prevent the UVB-Induced Suppression of the Contact Hypersensitivity Response in a Local Model of Contact Hypersensitivity

As it is well established that IL-12 acts to stimulate immune responses (30, 31) and silymarin enhances the levels of IL-12 in the skin and draining lymph nodes of UVB-irradiated mice, we investigated whether the silymarin-induced enhancement of the levels of IL-12 contributes to the prevention of the UVB-induced suppression of

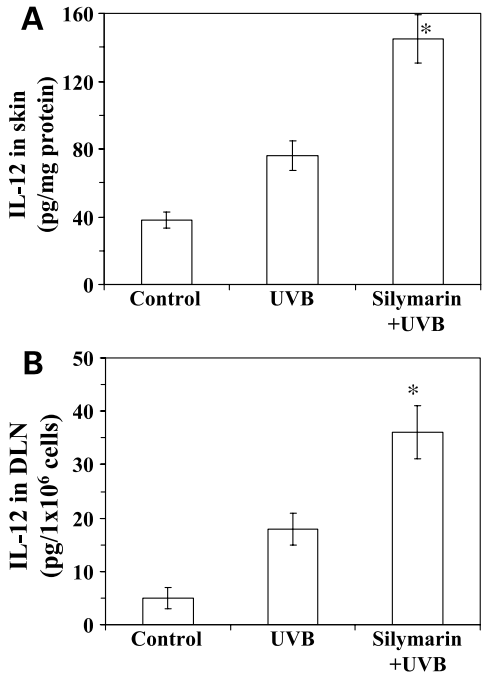

Figure 5. Silymarin increases the IL-12 concentration synergistically in UVB-exposed skin (**A**) and in the draining lymph node cells from UVB-exposed mice (**B**). The concentrations of IL-12 protein in skin homogenates and by the cells from lymph nodes were determined by ELISA. *Columns,* mean amount of IL-12 protein expressed either as pg/mg protein or pg/1 × 10^6 cells ($n = 5$ per group); *bars,* SD. Experiments were repeated once with identical results. *, $P < 0.005$, significant increase versus UVB alone control group.

the contact hypersensitivity response in mice. UVB exposure resulted in significant suppression of the local contact hypersensitivity response (76%; $P < 0.001$) to the contact sensitizer DNFB (Fig. 6, compare column 3 from the top with column 2 from the top), and silymarin treatment resulted in significant inhibition of the UVB-induced suppression of the contact hypersensitivity response in C3H/HeN mice (80%; $P < 0.001$; Fig. 6, column 4 from the top). I.p. injection of anti-IL-12 monoclonal antibody resulted in significant inhibition of the silymarin-induced prevention of UVB-induced suppression of contact hypersensitivity response ($P < 0.005$; Fig. 6, column 1 from the bottom) compared with the response in similarly treated mice that received rat IgG1 rather than anti-IL-12 (Fig. 6, column 4 from the top). This observation indicates that the prevention of UVB-induced suppression of contact hypersensitivity in mice by silymarin is mediated, at least in part, through the augmentation of the levels of IL-12.

Silymarin Does Not Prevent UVB-Induced Suppression of Contact Hypersensitivity Response in IL-12 KO Mice

As an alternative approach to establishing whether the prevention of UVB-induced immunosuppression by silymarin is mediated through enhancement of the levels of IL-12 in mice, we conducted experiments using IL-12 KO mice on a C3H/HeN background. Application of DNFB to

UVB-exposed skin did not induce sensitization (as determined by the ear swelling response) in either the IL-12 KO mice or their wild-type (WT) counterparts (Fig. 7A and B, column 4 from the top) but induced sensitization in the positive control group of mice that were not exposed to UVB radiation (column 2 from the top). It was also noticed that the DNFB-induced contact hypersensitivity response in IL-12 KO mice was significantly less (21%; $P < 0.05$) than WT mice, which may be due to absence of IL-12. Treatment of the WT mice confirmed that silymarin prevented UVB-induced suppression of contact hypersensitivity as shown by significant enhancement of the contact hypersensitivity ear swelling response on ear challenge (Fig. 7A, column 5 from the top). In contrast, treatment of IL-12 KO mice with silymarin did not prevent UVB-induced suppression of the contact hypersensitivity response to DNFB (Fig. 7B, column 5 from the top). Treatment with silymarin alone did not affect the DNFB-induced sensitization in the mice (Fig. 7A and B, column 3 from the top).

I.p. administration of recombinant IL-12 to the WT mice (Fig. 7A) and silymarin-treated or non-silymarin-treated IL-12 KO mice 3 hours before DNFB sensitization (Fig. 7B) resulted in an enhanced contact hypersensitivity response on DNFB treatment in WT and IL-12 KO mice as indicated by a robust ear swelling response (Fig. 7A, bottom column, and Fig. 7B, columns 1 and 2 from the bottom; $P < 0.005$). This robust ear swelling response was not observed in UVB-irradiated IL-12 KO mice that were (column 5 from the top) or were not (column 4 from the top) treated similarly. Thus, injection of IL-12 KO mice with IL-12 restored the responsiveness of the UVB-irradiated IL-12 KO mice to silymarin. The injection of IL-12 to both WT (Fig. 7A, bottom column) and IL-12 KO (Fig. 7B, bottom column) mice inhibits UVB-induced suppression of contact hypersensitivity response further confirmed the role of IL-12 in the enhancement of contact hypersensitivity response in UVB-irradiated mice. Taken together, these studies provide strong evidence that prevention of UVB-induced immunosuppression by silymarin is mediated, at least in part, through enhancement of the levels of IL-12.

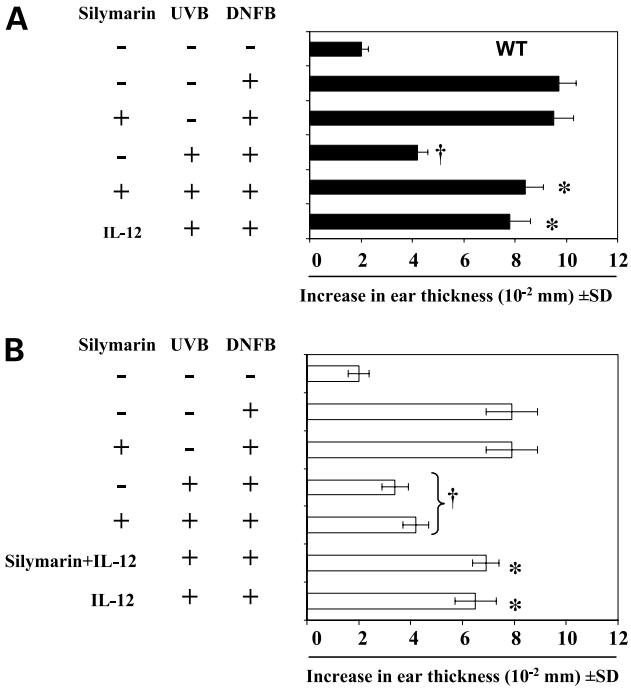

Figure 7. Silymarin prevents UVB-induced suppression of the contact hypersensitivity response in WT but not in IL-12 KO mice. The shaved backs of WT (**A**) or IL-12 KO (**B**) mice were exposed to UVB radiation (180 mJ/cm^2) with or without the prior treatment of silymarin (1 mg/cm^2). Three days later, the mice were sensitized with DNFB through UVB-exposed dorsal skin. Five days after sensitization, the mice were challenged by painting DNFB on the ear, and ear swelling was measured as detailed in Materials and Methods. Mice in **A** (bottom column) and **B** (columns 1 and 2 from the bottom) received 1,000 ng IL-12 i.p. 3 h before DNFB sensitization. *Columns,* mean change in ear thickness reported in mm $\times$ 10^{-2} (n = 5 per group); *bars,* SD. Injection of IL-12 in both WT and IL-12 KO mice inhibits UVB-induced suppression of contact hypersensitivity response to DNFB. The experiment was repeated twice with similar results. *, $P < 0.005$, significant sensitization versus silymarin + UVB; †, $P < 0.001$, significant inhibition versus positive control.

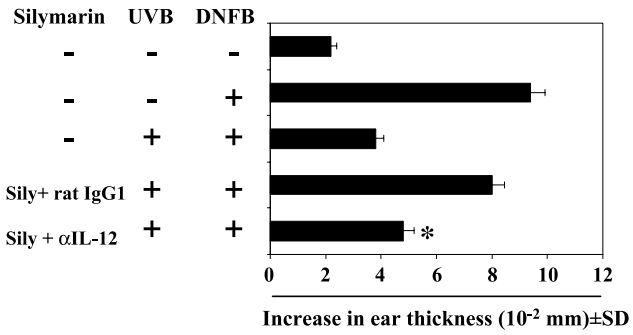

Figure 6. Administration of an anti-IL-12 monoclonal antibody *in vivo* inhibits the silymarin-induced prevention of the UVB-induced suppression of the local contact hypersensitivity response in mice. The mice received an i.p. injection of endotoxin-free anti-IL-12 or rat IgG1 (isotype control of anti-IL-12) at 2 $\times$ 500 ng/mouse diluted in sterile endotoxin-free saline 24 and 3 h before DNFB sensitization. *Columns,* mean change in ear swelling response in each group (n = 5 per group); *bars,* SE. Experiments were repeated once with identical results. *Sily,* silymarin. *, $P < 0.005$, significant inhibition versus positive control and silymarin + rat IgG1 + UVB – treated groups.

Discussion

We have shown previously that topical application of silymarin inhibits UVB radiation-induced inflammatory responses, oxidative stress, and induction of photocarcinogenesis in mice (2, 22, 25). It also has been shown that silibinin (a major constituent of silymarin) inhibits UVB-induced skin photodamage, including the inhibition of photocarcinogenesis in mice whether it is applied topically before or after UVB irradiation or given in the diet (33). As UVB-induced immunosuppression has been implicated in the development of photocarcinogenesis, we examined the efficacy of silymarin on UVB-induced immunosuppression using local and systemic models of contact hypersensitivity in C3H/HeN mice. The results presented here show that

topical application of silymarin inhibits UVB-induced suppression of contact hypersensitivity response to DNFB in both local and systemic models of contact hypersensitivity. These data provide a first line of evidence that prevention of photocarcinogenesis by silymarin may be, at least in part, due to the prevention of UVB-induced immunosuppression in mice.

Similar effects were observed when the effects of silibinin on UVB-induced suppression of the contact hypersensitivity response were examined using identical experimental conditions and models. In the contact hypersensitivity model used in these studies, silymarin seemed to be more effective in preventing immunosuppression than silibinin (Fig. 1), although this difference did not reach statistical significance. It is possible that silymarin could prove to be a better chemopreventive agent than silibinin because of the potential synergy among all the constituents of this plant product. Further, although silymarin was effective in preventing UVB-induced immunosuppression under normal levels of UVB exposure, it failed to prevent immunosuppression induced by an acute high dose of UVB (1,000 mJ/cm^2). This failure most likely can be attributed to the severity of the cellular damage incurred using this dose of UVB.

In terms of the mechanisms by which silymarin mediates the inhibition of UVB-induced immunosuppression, our data show that treatment with silymarin inhibits the UVB enhancement of IL-10 levels both in the skin and in the draining lymph nodes. It has been shown that i.p. administration of IL-10 inhibits the sensitization of mice to trinitrophenyl-coupled spleen cells in an assay of delayed-type hypersensitivity (36) and that i.p. injection of IL-10 resulted in a significant suppression of the ear swelling response in a model of contact hypersensitivity, suggesting that IL-10 has the ability to block the effector phase of contact hypersensitivity *in vivo*. Furthermore, it has been shown that administration of neutralizing antibodies to IL-10 largely inhibited the ability of UV radiation to suppress sensitization to alloantigens (35). In agreement with these observations, our data suggest that prevention of UVB-induced immunosuppression by silymarin may be mediated, at least in part, through the inhibition of UVB-induced increase in IL-10 production in the draining lymph nodes and the skin.

We also found that silymarin treatment increased the production of IL-12 in the skin and draining lymph nodes of UVB-exposed C3H/HeN mice. The role of these enhanced levels of IL-12 production in the silymarin inhibition of the UVB-induced immunosuppression was confirmed using a local contact hypersensitivity model, which showed that i.p. injection of anti-IL-12 antibody before sensitization resulted in the silymarin-treated mice exhibiting UVB-induced suppression of the contact hypersensitivity response to DNFB. This observation was further supported by our experiments conducted using IL-12 KO mice and their WT counterparts (C3H/HeN) in which we found that topical application of silymarin failed to prevent UVB-induced immunosuppression in the IL-12 KO mice but prevented it in WT mice. Further, the i.p. injection of recombinant IL-12 to UVB-exposed WT and IL-12 KO mice both restored contact hypersensitivity response in the mice, which support the evidence that IL-12 plays a crucial role in prevention of UVB-induced immunosuppression in mice. The immunostimulatory effects of IL-12 have been shown in an *in vivo* system (30, 31) and IL-12 has been shown to play a role *in vivo* as a mediator and adjuvant for the induction phase of the contact hypersensitivity response (30). Contact hypersensitivity seems to be a Th1-type cell-mediated immune response (38), and the Langerhans cells, which act as critical epidermal antigen-presenting cells in the induction phase of contact hypersensitivity (39), have been reported to produce IL-12. After UV exposure, the antigen-presenting cells present in the skin migrate to the regional lymph nodes and initiate sensitization. It is possible that silymarin treatment enhances the levels of IL-12 in the draining lymph nodes by increasing the number of antigen-presenting cells that migrate from the skin to the regional lymph nodes in UVB-irradiated mice.

IL-12 stimulates the development and function of T cells, particularly the development of Th1-type cells by stimulating the production of IFN-γ (40–42). I.p. injection of IL-12 prevents UV-induced suppression of contact hypersensitivity (43) and overcomes UV-induced hapten-specific tolerance in mice (44). In the contact hypersensitivity model used in the present study, the silymarin-induced increases in the levels of IL-12 in the draining lymph nodes of the UVB-irradiated mice could tilt the immune response in favor of the development of Th1-type cells. Taken together with the effect of silymarin on the production of IL-10, the silymarin-induced shift in the cytokine balance of IL-10 and IL-12 seems to be a potential mechanism by which silymarin may reverse or inhibit UVB-induced suppression of contact hypersensitivity in mice. Similar immunoprotective effects of other polyphenols, such as (−)-epigallocatechin-3-gallate from green tea (20), have been observed and they seem to share similar effector mechanisms.

The data from the present study indicate clearly that inhibition of IL-10 production and induction of IL-12 by silymarin in UVB-exposed mice may contribute to the inhibition of UVB-induced suppression of contact hypersensitivity response in mice. The mechanisms by which silymarin and the other polyphenols exert these effects are still not clear, however, and require further study. Together, our data suggest for the first time that silymarin, a plant flavonoid, can protect mice from UVB-induced immunosuppression and that this effect may be associated with the ability of silymarin to protect mice from photocarcinogenesis.

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