Silibinin prevents ultraviolet radiation-caused skin damages in SKH-1 hairless mice via a decrease in thymine dimer positive cells and an up-regulation of p53-p21/Cip1 in epidermis

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Non-melanoma skin cancer (NMSC) accounts for >1 million new cases each year in the US alone suggesting that more approaches are needed for its prevention and control. Earlier studies by us have shown that silymarin (a crude form of biologically active silibinin with some other isomers), isolated from milk thistle, affords strong protection against ultraviolet (UV) radiation-induced NMSC in SKH-1 hairless mice; however, the molecular mechanisms of its efficacy are not known. Here, we assessed the effect of silibinin on UV-induced DNA damage and p53-p21/Cip1 accumulation, and their roles in UV-induced cell proliferation and apoptosis in SKH-1 hairless mouse epidermis. Topical application of silibinin prior to, or immediately after, UV irradiation resulted in a very strong protective effect against UV-induced thymine dimer positive cells in epidermis accounting for 76–85% (P < 0.001) inhibition. In other studies, silibinin treatment resulted in a further up-regulation of p53 by -1.6-fold (P < 0.001) together with an increase (~2-fold, P < 0.001) in p21/Cip1 protein levels. Proliferative cell nuclear antigen staining showed that silibinin pre- or post-topical application significantly inhibits (40–52 and 20–40%, respectively, P < 0.001) UV-induced epidermal cell proliferation. In addition, silibinin strongly decreased UV-caused terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling-positive apoptotic/sunburn cell formation (P < 0.001). These findings suggest that silibinin affords strong protection against UV-induced damage in epidermis by a decrease in thymine dimer positive cells and an up-regulation of p53-p21/Cip1 possibly leading to an inhibition in both cell proliferation and apoptosis. Comparable effects of silibinin following its pre- or post-UV application suggest that mechanisms other than sunscreen effect are operational in silibinin efficacy against UV-caused skin damages.

Introduction

Ultraviolet (UV) radiation, in the range of 290–320 and 320–400 nm for UVB and UVA wavelengths, respectively, from the sunlight is the major etiologic factor for the non-melanoma skin cancer (NMSC) (1). In addition to its carcinogenic activity, UV also causes photoaging, immunosuppression and sunburn (2–4). There is high morbidity with NMSC, and the associated medical cost is estimated to be over $500 million/year. In spite of skin cancer awareness in recent years, the prediction is a further increase in NMSC cases possibly because of several outdoor activities and depletion in atmospheric ozone (5). This emphasizes the importance of developing additional strategies that could control and prevent solar UV-caused NMSC. Although the usage of sunscreens has been an important protection strategy, further approaches are needed to more effectively protect human skin against UV-caused damages as well as NMSC (6,7).

Chemoprevention of cancer is a novel and more effective means of cancer control where naturally occurring agents are considered as a less toxic and more effective approach in controlling various human malignancies including NMSC (8–12). For example, oral administration or topical application of green tea and black tea, with or without caffeine, has been shown to strongly prevent UV-induced skin cancer in mouse skin (13), inhibit skin carcinogenesis in UV-exposed high-risk mice (12), and increase apoptotic sunburn cells by inducing p53 and p21/Cip1 (14). Earlier studies by us have shown that silymarin (a crude form of biologically active silibinin with some other isomers), isolated from milk thistle [Silybum marianum (L.) Gaertn] plant, affords strong protection against: (i) UV-induced tumor initiation, promotion and complete carcinogenesis in SKH-1 hairless, and (ii) chemical carcinogenesis in SENCAR mouse skin (15). Completed studies by us have also shown that silymarin (a crude form of biologically active silibinin with some other isomers), isolated from milk thistle [Silybum marianum (L.) Gaertn] plant, affords strong protection against: (i) UV-induced tumor initiation, promotion and complete carcinogenesis in SKH-1 hairless, and (ii) chemical carcinogenesis in SENCAR mouse skin (15–19). Completed studies by us have also shown that silymarin inhibits UV- and chemical tumor promoter-induced cellular and biochemical events associated with skin tumor promotion (15–19); however, the mechanism of its efficacy against UV-caused damages leading to skin tumor initiation is not known, which was explored in the present investigation.

UV radiation is known to exert its tumor initiating effects primarily through the formation of cyclobutane pyrimidine dimers and 6-4 photoproducts (20) in DNA, as well as via formation of reactive oxygen species that subsequently lead to the damage of DNA and other major cellular targets (21). The UV-damaged cell responds to these alterations either by enhanced DNA repair or by inducing apoptotic death, if the damage is too severe; failure to do so, however, could lead to the formation of an initiated cell (22). One important cellular response occurring after UV exposure and/or UV-caused DNA damage is phosphorylation of p53 at various serines that increases its half-life causing its accumulation (23). This also increases the transcriptional activity of p53 leading to the synthesis of its target genes such as p21/Cip1 that helps the cells in: (i) arresting DNA synthesis and allowing more time for DNA repair, or (ii) modulating bax levels causing apoptosis (24,25). Consistent with above reports, in the present study, we assessed the effect of topical application of silibinin on UV-induced DNA damage in terms of thymine dimer positive

Abbreviations: H&E, hematoxylin and eosin; NMSC, non-melanoma skin cancer; PCNA, proliferating cell nuclear antigen; UV, ultraviolet; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling.

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cells, cell proliferation and apoptosis as well as the role of p53 and p21/Cip1 molecules in these UV-induced early adaptive changes, in female SKH-1 hairless mouse epidermis.

Materials and methods

Animals and UV light source

Female SKH-1 hairless mice (5 weeks old) were obtained from Charles River Laboratories (Wilmington, MA) and maintained in animal house facility at the University of Colorado Health Sciences Center. Animals were fed with Purina Chow and water ad libitum for a week before the start of the experiments. The UV light source was a bank of four FS-40-T-12-UVB sunlamps equipped with a UVB Spectra 305 Dosimeter (Daavlin, Bryan, OH), which emitted ~80% radiation in the range of 280–340 nm with a peak emission at 314 nm as monitored with a SEL 240 photodetector, 103 filter and 1008 diffuser attached to an IL1400A Research Radiometer (International Light, Newburyport, MA). The UVB irradiation doses were also calibrated using IL1400A radiometer. Mice were exposed to UV irradiation as reported earlier (15) with a distance of 23 cm between the light source and the target skin. Silibinin used in the study was obtained commercially from Sigma Chemical Company (St Louis, MO), and was analyzed by HPLC as a pure agent as reported earlier (17).

Experimental design

Animals were divided into five groups containing five animals in each group for each time-point of the study as follows: (i) unexposed and untreated control mice, (ii) animals topically applied with 9 mg silibinin in 200 μl acetone/ mouse, (iii) animals irradiated once with 180 mJ/cm² UV dose, (iv) animals topically applied with 9 mg silibinin in 200 μl acetone/mouse 30 min prior to identical UV exposure as in group 3, and (v) animals topically applied with 9 mg silibinin in 200 μl acetone/mouse immediately after identical UV exposure as in group 3. Animals were killed after UV exposure at various time points, and dorsal skin was collected, fixed in 10% formalin for 8–10 h at 4°C and processed for immunohistochemical analyses.

Immunohistochemical and hematoxylin and eosin (H&E) staining analyses

Skin samples were processed conventionally before paraffin embedding. Briefly, skin samples were dehydrated in 70, 95 and 100% ethanol, cleared in xylene and embedded in paraffin. Serial, 4 μm sections were cut and used for all immunohistochemical and H&E analyses. The sections were deparaffinized, rehydrated with water and used for measurement of thymine dimer, p53, p21/Cip1, proliferating cell nuclear antigen (PCNA), terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) and apoptotic sunburn positive cells. All samples were coded and evaluated by two investigators in a blinded manner and the mean ± SE values were obtained from the evaluation of multiple fields in each group. Five to ten representative fields were counted for each mouse at 400× magnification, and the data represent the results from at least five mice in each group.

Measurement of thymine dimer positive cells

Thymine dimer positive cells in epidermal layer were measured as described earlier (26). Briefly, endogenous peroxidase activity was blocked by 10 min incubation with 3% hydrogen peroxide, and slides were then incubated with 0.125% trypsin for 10 min at 37°C and then with 1 N HCl for 30 min at room temperature. The sections were then blocked with 5% goat serum for 10 min and incubated with peroxidase-conjugated monoclonal anti-thymine dimer antibody (Kamiya Biomedical Company, Seattle, WA) for 1 h at room temperature followed by 30 min incubation with HRP-conjugated streptavidin. Color development was achieved by incubation with DAB for 10 min at room temperature. The sections were counterstained with Harris Hematoxylin, dehydrated and mounted.

TUNEL staining for apoptotic cells

Apoptotic cells were detected using the DeadEnd Colorimetric TUNEL system (Promega, Madison, WI) following manufacturer’s protocol with some modifications. In brief, tissue sections after deparaffinization and re-hydration, were permeabilized with Proteinase K (30 μg/ml) for 1 h at 37°C. Thereafter, the sections were quenched of endogenous peroxidase activity using 3% hydrogen peroxide for 10 min. After thorough washing with 1 × PBS, sections were incubated with equilibration buffer for 10 min, and then TdT reaction mixture was added to the sections, except for the negative control, and incubated at 37°C for 1 h. The reaction was stopped by immersing the sections in 2 × saline-sodium citrate buffer for 15 min. Sections were then added with streptavidin-HRP (1:500) for 30 min at room temperature, and after repeated washings, sections were incubated with substrate DAB until color development (~5–10 min). Sections were then mounted after re-hydration and observed under 400× for TUNEL-positive cells.

Measurement of apoptotic sunburn cells

Skin sections were stained conventionally with H&E as mentioned earlier (15) for the identification of sunburn cells. Apoptotic cells are morphologically distinct due to cell shrinkage and nuclear condensation that stain darker by H&E. Dark stained cells were scored in 5 random fields/sample and the percentage/field was calculated.

Immunohistochemical and statistical analyses

All the microscopic immunohistochemical analyses were done using Zeiss Axioscop 2 microscope (Carl Zeiss, Jena, Germany). Pictures were taken by a digital camera attached to the microscope. Karnovsky’s fixative (2.5% glutaraldehyde and 2.5% formaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4) was used for collecting fresh samples. The samples were fixed in the Karnovsky’s fixative for 1 h at room temperature followed by 1 N HCl for 30 min at room temperature. Thereafter, the sections were quenched of endogenous peroxidase activity using 3% hydrogen peroxide for 10 min. After thorough washing with 1 × PBS, sections were incubated with equilibration buffer for 10 min, and then TdT reaction mixture was added to the sections, except for the negative control, and incubated at 37°C for 1 h. The reaction was stopped by immersing the sections in 2 × saline-sodium citrate buffer for 15 min. Sections were then added with streptavidin-HRP (1:500) for 30 min at room temperature, and after repeated washings, sections were incubated with substrate DAB until color development (~5–10 min). Sections were then mounted after re-hydration and observed under 400× magnification for TUNEL-positive cells.

Results

Silibinin inhibits UV-induced DNA damage in SKH-1 hairless mouse epidermis

Formation of thymine dimers is one of the characteristics of UV-induced DNA damage, and occurs three times more often as compared with other photoproducts (27). Based on an earlier study showing that thymine dimer formation occurs as early as 5 min and peaks at 1 h after UV exposure (26), we assessed the effect of silibinin on thymine dimer positive cells in mouse epidermis 1 h after a single UV exposure at 180 mJ/cm² dose. As shown in Figure 1, compared with unexposed control mice, UV irradiation resulted in a very strong immunostaining for thymine dimer in the mouse epidermis; however, topical application of silibinin prior to or immediately after (immunostaining data not shown) UV exposure showed a remarkably reduced immunostaining for UV-induced thymine dimer positive cells in epidermis (Figure 1A–C). In quantitative analysis, UV exposure resulted in 78 ± 2% thymine dimer positive cells in the epidermis while no such cells were observed in unexposed controls (Figure 1D). Pre- or post-treatment application of silibinin, to UV exposure, resulted in only 12 ± 1 and 19 ± 1% thymine dimer positive cells, accounting for 85 and 76% inhibition (P < 0.001), respectively (Figure 1D). These data clearly suggest that preventive efficacy of silibinin against UV-caused skin tumor initiation could be, in part, via an inhibition in UV-induced DNA damage.
Silibinin up-regulates UV-induced activation of p53-p21/Cip1 cascade

p53 is known to play a crucial role in DNA damage response where it arrests cell proliferation when the damage is mild or induces apoptosis when the damage is severe (28–30). Based on our findings showing that silibinin strongly reduces UV-caused thymine dimer positive cells, we next focused our efforts on assessing the effect of silibinin on UV-caused p53 accumulation. As shown in Figure 2, compared with a
negligible immunostaining for p53 in unexposed mouse epidermis, a large number of p53-positive cells were observed in the epidermis at 8 h after a single UV exposure of mice. These results are consistent with an earlier report showing p53 accumulation in mouse epidermis following 8–12 h of UV exposure at an identical dose used in the present study (26). Silibinin topical application prior or immediately after UV exposure, however, resulted in a further increase in p53-positive cells (Figure 2A–C). Overall, in quantitative analysis, compared with 0.81 ± 0.32% p53-positive cells in unexposed control epidermis, 8 h after UV exposure resulted in 23.8 ± 1.8% p53-positive cells, which were further increased by pre- or post-topical application of silibinin to 36.4 ± 2.1 and 36.9 ± 1.2% (P < 0.001) p53-positive cells, respectively, accounting for ~1.6-fold increase (Figure 2D).

UV-induced increase in p53 protein accumulation is known to enhance the synthesis of p21/Cip1 protein, which plays a crucial role in the adaptive responses after UV exposure of the skin by inhibiting cell proliferation (29). Based on our finding that silibinin further enhances UV-induced p53-positive cells in epidermis, same samples were also analyzed for p21/Cip1-positive cells. Compared with almost no immunostaining in unexposed controls, a strong immunoreactivity for p21/Cip1-positive cells was observed in the epidermis at 8 h after a single UV irradiation at 180 mJ/cm² dose, which, similar to p53 results, was further enhanced by pre- or post-application of silibinin (data not shown). In quantitative estimates, UV exposure showed 9.9 ± 0.9% p21/Cip1-positive cells in epidermis compared with 0.34 ± 0.09% in unexposed controls; however, pre- or post-UV silibinin treatment resulted in 21.0 ± 1.8 and 18.2 ± 1.8% p21/Cip1-positive cells, respectively, accounting for ~2-fold increase (P < 0.001) over UV-alone group (Figure 2E). Together, the data shown in Figures 1 and 2 clearly suggest that silibinin reduces UV-caused thymine dimer positive cells and further increases UV-activated p53-p21/Cip1 cascade in mouse epidermis, which might be involved in an overall protection against UV-caused biological responses such as cell proliferation and apoptosis induction. Accordingly, we next assessed the effect of silibinin on these two biological endpoints.

Silibinin inhibits UV-induced cell proliferation in SKH-1 hairless mouse skin

As shown in Figure 3, compared with unexposed controls, a single UV irradiation at the dose of 180 mJ/cm² resulted in a strong PCNA staining within 1 h of the irradiation; however, topical application of silibinin prior to or immediately after UV irradiation resulted in a strong inhibition in UV-induced PCNA-positive cells in the epidermis (Figure 3A–C). A quantitative analysis of the PCNA immunostaining showed that UV irradiation caused 22.4, 18.4, 19.6 and 16.1% PCNA-positive cells at 1, 4, 8 and 12 h post-UV irradiation as compared with 3.8% PCNA-positive cells in un-irradiated control epidermis, respectively (Figure 3D). However, topical application of silibinin at 9 mg dose in 200 μl acetone/mouse 30 min prior to UV exposure and killed 1 h thereafter. Skin was removed, fixed, paraffin-embedded and processed for immunohistochemical analysis of PCNA as described in the Materials and methods. Under identical experimental conditions and treatments, mice were killed at 1, 4, 8 and 12 h after UV exposure, and skin tissue samples were subjected to PCNA staining as described in the Materials and methods. Percent PCNA-positive cells were calculated as mean of five randomly selected fields (400×) from each skin sample. Data shown are mean ± SE of 25 fields/5 mice/group. Sb, Silibinin.

Silibinin inhibits UV-induced apoptotic and sunburn cell formation

Consistent with its effect on a reduction in UV-caused thymine dimer positive cells, and a further increase in UV-activated p53-p21 accumulation, silibinin treatment prior to or immediately after UV exposure also significantly inhibited UV-induced apoptosis (Figure 4A–D). As observed by TUNEL staining, compared with 2.9 ± 0.73% (Figure 4A) apoptotic

![Fig. 3. Silibinin inhibits UV-induced cell proliferation as observed by PCNA immunostaining.](image-url)
cells in unexposed controls, a single UV irradiation resulted in 30.5 ± 1.34% (Figure 4B) TUNEL-positive apoptotic cell population. However, pre- or post-application of silibinin showed only 9.0 ± 0.81 (Figure 4C) and 20.6 ± 1.08% apoptotic cells accounting for 70 and 32% decrease (P < 0.001), respectively (Figure 4A–D). UV-caused apoptotic/sunburn cell formation was further confirmed by H&E staining, in which exposure of mouse skin to UV resulted in 10.2 ± 0.94% (P < 0.001) sunburn cells as compared with 0.88 ± 0.16% in un-irradiated control (Figure 4E). Consistent to TUNEL staining results, the UV-caused sunburn cell formation was inhibited significantly to 5.0 ± 0.37 and 6.8 ± 0.62% (P < 0.001) sunburn cells, respectively, by pre- or post-topical application of silibinin (Figure 4E).

Discussion

The central finding in the present study is that silibinin protects SKH-1 hairless mouse skin from UV-induced DNA damage, and that UV-caused cell proliferation and apoptotic sunburn cell formation are prevented by silibinin possibly via further induction in p53-p21/Cip1 cascade. It is also important to emphasize here that we employed two different silibinin topical application protocols in which silibinin was applied 30 min prior to UV exposure or immediately thereafter. This approach was important to dissect out the silibinin efficacy against UV-caused skin damage independent of its sunscreen effects. The findings reported here clearly support our conclusion that silibinin efficacy against UV-caused skin damage and photocarcinogenesis is not only due to its sunscreen effect.

One of the most important characteristics of UV-caused carcinogenesis is DNA damage and mutagenesis, and thymine dimers are known as ‘hot spots’ of UV mutagenesis (20). In our study, silibinin treatment resulted in a remarkable decrease in UV-caused thymine dimer positive cells. Analysis of the mismatch repair enzyme MSH2 showed that silibinin treatment does not result in an increase in MSH2 levels (data not shown). This suggests the possible involvement of other mismatch repair enzymes. Further, there are some reports showing that DNA mismatch repair system is inactivated by oxidative stress (31). Strong anti-oxidant properties of silibinin reported earlier by us in mouse skin studies (15,16,18) suggest that suppression of oxidative stress by silibinin could be one of the possible mechanisms that resulted in the activation of repair enzymes much earlier as compared with UV alone; thereby it is possible that thymine dimers were removed much earlier in silibinin-treated animals than 1 h, the time point used in our study. More studies are needed in future as a function of time employing time points earlier than 1 h, specifically between 5 and 60 min, to assess whether silibinin causes a faster repair of UV-caused DNA damage ultimately leading to a strong reduction in thymine dimer positive cells in epidermis as observed in the present study at 1 h after UV exposure of SKH-1 mouse skin. The other possibilities could be that silibinin protects epidermal cells from UV-caused thymine dimer positive cells by modulating DNA repair enzymes other than MSH2 and/or by an alteration in ATM/ATR pathways. In this regard, recent studies have shown the role and involvement of ATM/ATR in UV-induced DNA damage (32), and accordingly future studies are needed to assess silibinin effect on these pathways as an upstream response for its efficacy against UV-caused thymine dimer positive cells in epidermis.

p53 up-regulation following UV irradiation is known to protect the cells from UV-caused cellular damage/s via several mechanisms including an induction in cell cycle arrest via transcriptional activation of p21/Cip1, or by inducing apoptosis through an activation of apoptotic proteins bax, Fas/Apo-1, DR5 or by down-regulation of anti-apoptotic proteins such as...
bcl-2 and cellular inhibitor of apoptosis protein 2 (c-IAP2) (23,29). Some chemopreventive agents have been shown to selectively induce apoptosis of UV-damaged cells by up-regulation of p53 (14,33). Earlier reports have shown that the level of p53 accumulation depends on the extent of DNA damage and that lower levels of p53 correspond to cell cycle arrest (34). In our study, silibinin treatment resulted in a further increase in UV-induced p53 accumulation with a concomitant increase in p21/Cip1 protein levels, which is in accord with the inhibition of UV-induced cell proliferation and apoptosis by silibinin, suggesting their possible role in cell growth inhibition rather than apoptosis induction (Figure 5).

UV-induced increase in cell proliferation is an early necessary event associated with UV-caused carcinogenesis that helps the initiated cells to proceed further into cell cycle (24); this response, however, could be prevented by arresting the cells at G1 or S phase of the cell cycle (35). We observed that UV-induced PCNA-positive cells were strongly inhibited by silibinin treatment. It has been reported that UV-induced cell proliferation reaches the maximum level as early as 1 h after exposure, and we observed that silibinin treatment showed a strong inhibitory effect even at that early time point. In addition, the inhibitory effect of silibinin on UV-caused cell proliferation was sustained as observed up to 12 h after exposure. These results suggest that inhibiting cell proliferation could be one of the mechanisms by which silibinin protects damaged cells from entering the cell cycle, thereby providing damaged cells additional time for repair and preventing their entry into apoptotic pathway in case the damage is severe. This suggestion is in accord with the observation that silibinin treatment further up-regulates p21/Cip1 levels, which is known to inhibit cell proliferation by a direct binding with PCNA (36).

An earlier study by us has shown that topical application of silymarin strongly protects against UV-caused sunburn cell formation in SKH-1 hairless mice (15). The results of the present study showing the inhibitory effect of silibinin on UV-caused sunburn cell formation further support the previous finding. In addition, the TUNEL staining results in the present study are consistent with an early report regarding apoptotic nature of the sunburn cells (37).

Use of conventional chemical sunscreens has been an effective measure in protecting skin against UV-caused damages and NMSC; several cancer chemopreventive agents, however, have also been shown to protect cells from UV by acting as a sunscreen (10,38,39). However, sunscreen usage as an only measure is not adequate because of infrequent use, incomplete protection and associated toxicity (7). In this regard, topical application of antioxidants has been shown to be beneficial against skin cancer as well as associated hazards such as photoaging. It is important to emphasize here that silibinin is a flavonoid antioxidant with no known toxicity in both animal and human studies. Further, since the chemopreventive efficacy of silibinin has been well studied and established in several epithelial cancer models (16–18,40,41), it could be a potent chemopreventive agent against NMSCs as well as UV-caused skin damages and photoaging. In the present study, as pretreatment of silibinin showed a moderately better protective effect (than its post-treatment) against some of the UV-caused alterations in SKH-1 mouse epidermis, a sunscreen effect may be an additional factor in silibinin-afforded protection.

In conclusion, the results of the present study suggest that silibinin protects SKH-1 hairless mouse epidermis from UV-induced cell proliferation and apoptosis through a reduction in DNA damage and via an activation of p53-p21/Cip1 cascade. These results warrant the development of silibinin as a pharmacological non-toxic chemopreventive agent against human NMSC and photoaging. In addition, more mechanistic studies are needed in future to define the protective effect of silibinin on UV-induced DNA damage and apoptosis, and their biological significance in an overall efficacy of silibinin against UV-caused skin damages and photocarcinogenesis.

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Silibinin protects mouse skin against UV damage

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