Differential effect of silibinin on E2F transcription factors and associated biological events in chronically UVB-exposed skin versus tumors in SKH-1 hairless mice

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Abstract
UVB radiation–induced DNA damage in skin activates cellular pathways involved in DNA repair, cell cycle regulation, and apoptosis, important events that prevent conversion of damaged skin cells into cancer. We reported recently the efficacy of silibinin against photocarcinogenesis along with altered molecular events in tumors (Cancer Research, 64:6349–56, 2004). The molecular and biological events modulated by silibinin in chronically UVB-irradiated skin leading to cancer prevention, however, are not known. Herein, we describe effect of silibinin on skin 15 and 25 weeks after UVB exposure and compared them with molecular alterations in skin tumors. UVB decreased E2F1 but increased E2F2 and E2F3 protein levels in skin, and these were reversed by silibinin treatment. Silibinin-induced E2F1 was accompanied by an inhibition of apoptosis and decreases in p53 and cyclin-dependent kinase inhibitors. Silibinin-caused decrease in E2F2 and E2F3 was accompanied by reduced levels of cyclin-dependent kinases, cyclins, CDC25C, and mitogen-activated protein kinases and Akt signaling and inhibition of cell proliferation. In tumorigenesis protocols, topical or dietary silibinin significantly inhibited tumor appearance and growth. As opposed to UVB-exposed skin, UVB-induced tumors showed elevated levels of E2F1, but these were reduced in silibinin-treated tumors without any effect on E2F2 and E2F3. Contrary to the inhibition of apoptosis and p53 expression in UVB-exposed skin cells, silibinin increased these variables in tumors. These differential effects of silibinin on E2F1 versus E2F2 and E2F3 and their associated molecular alterations and biological effects in chronic UVB-exposed skin suggest their role in silibinin interference with photocarcinogenesis. [Mol Cancer Ther 2006;5(8):2121–29]

Introduction
Skin is the largest organ in the body, covering an extensive surface area permanently exposed to environmental stresses. One of the important functions of the skin is to protect against UVB radiation capable of introducing genetic mutations that cause both melanoma and nonmelanoma skin cancers. UVB radiation causes many detrimental effects, such as sunburn, ocular damage, photoaging, immune suppression, DNA damage, and skin cancer. Each year, >106 new cases of nonmelanoma skin cancer are diagnosed in the United States (1). The social and economic burden of treating this disease has increased sharply and ranks among the top five medical expenditures (2). Surgical and medical treatments of nonmelanoma skin cancer are difficult because of a high recurrence rate, the occurrence of multiple lesions, and location of tumors on head, neck, and outer arms (2). These limitations suggest the need for additional approaches to protect skin against UVB-caused cellular damages and nonmelanoma skin cancer (3, 4).

UVB-caused skin damage and tumorigenesis depend on the ability of cells to detect and repair DNA damage, regulate cell cycle division, and execute efficient apoptosis (4). A tight coupling between factors involved in regulating cell cycle checkpoints and DNA damage repair allows damaged cells to pause at the G1-S or G2-M boundaries to prevent replication of damaged DNA (5). Whereas DNA repair pathways restore DNA integrity, damage that is severe and cannot be repaired causes cells to be removed from the cycling population by cell cycle arrest (1). Critical mediators of these events include retinoblastoma tumor suppressor protein and E2F transcription factors, which regulate many downstream target genes involved in cell cycle progression [e.g., cyclin A, cyclin E, CDC2, and cyclin-dependent kinase (CDK) 2] and DNA replication [e.g., proliferating cell nuclear antigen (PCNA); refs. 6–8]. The antiapoptotic activity of E2F1 is an important response to UVB-induced DNA damage because DNA repair is stimulated and apoptosis suppressed (9, 10), suggesting that targeting these molecules could be a mechanism-based approach to protect skin against UVB-caused damage and photocarcinogenesis.

Dietary phytochemicals, such as silibinin, prevent UVB-induced skin damage and cancer in preclinical and clinical studies (3, 4). Silibinin is a major bioactive flavonolignan (Fig. 1) present in milk thistle (Silybum marianum) extract.
that has been used as a traditional medicine for ~2,000 years to treat various liver conditions (4). Silibinin prevents tumorigenesis, inhibits cell growth via a cell cycle arrest, and induces differentiation or apoptosis in many human epithelial cancer cell types, including skin, prostate, breast, cervical, bladder, colon, and lung (4). We reported recently the efficacy of silibinin against photocarcinogenesis together with alterations in molecular events related to cell cycle progression and mitogenic and apoptotic pathways within tumors (11). During acute UVB exposure, silibinin inhibits the activation of mitogen-activated protein kinases (MAPK) and Akt, induces p53 and Cip1/p21 expression, and suppresses DNA damage in mouse skin (12, 13). However, these molecular and biological events modulated by silibinin in chronically UVB-irradiated skin that might be responsible for its skin cancer preventive efficacy are not known. This study on an SKH-1 hairless mouse model was designed to address whether silibinin treatment alters that key cell cycle regulatory pathway mediated by E2F transcription factors. These could mediate the biological responses during chronically UVB-exposed mouse skin that leads to the preventive efficacy of silibinin against photocarcinogenesis.

Materials and Methods

Animals, Diet, and UVB Source

Female SKH-1 hairless mice (5 weeks old) were purchased from Charles River Laboratories (Wilmington, MA). Silibinin was obtained commercially from Sigma Chemical Co. (St Louis, MO) and, on analysis by high-pressure liquid chromatography, shown to be a pure agent (12). A silibinin diet (1%, w/w) was prepared commercially (Dyets, Inc., Bethlehem, PA). The UVB light source consisted of four FS40T12-UVB sunlamps equipped with a UVB Spectra 305 Dosimeter (Daavlin Co., Bryan, OH). This emits 80% radiation within 280 to 340 nm, with a peak at 314 nm as monitored with SEL 240 photodetector, 103 filter, and 1008 diffuser attached to IL1400A Research Radiometer (11).

Experimental Design

The effect of topical or oral silibinin was evaluated during chronic UVB (180 mJ/cm², 2 days/wk for 15 and 25 weeks) exposure in female SKH-1 hairless mice. Treatment groups (20 mice each) were as follows: (a) sham-irradiated or untreated control (controls), (b) topically treated with 9 mg silibinin in 200 µL acetone/mouse, (c) fed 1% silibinin in diet, (d) irradiated with 180 mJ/cm² UVB, (e) topically applied with 9 mg silibinin in 200 µL acetone/mouse 30 minutes before UVB exposure, (f) topically applied with 9 mg silibinin in 200 µL acetone/mouse immediately after UVB exposure, and (g) fed 1% silibinin in diet and irradiated with same dose of UVB. These treatment protocols were based on our earlier studies showing silibinin efficacy against photocarcinogenesis and acute UVB exposure–caused changes in mouse skin (11–13). Body weight and diet consumption were recorded throughout the experiment. Mice were sacrificed when tumors first appeared (15 weeks after UVB) and when all the mice in groups d to g had tumors (25 weeks after UVB). In the tumor study, we measured the incidence, multiplicity, and volume of tumors (11). At the end of the 15th and 25th weeks, chronically UVB-exposed skin without visible tumors and skin tumors (only at the end of 25th week) were fixed in buffered formalin for immunohistochemical analyses or flash frozen in liquid nitrogen and analyzed by immunoblotting.

Tissue Sectioning and Immunohistochemical Staining

Normal skin (control groups), chronically UVB-exposed skin, and skin tumors were fixed in 10% phosphate-buffered formalin for 10 hours at 4°C, dehydrated in ascending concentrations of ethanol, cleared with xylene, and embedded in PolyFin (Triangle Biomedical Sciences, Durham, NC). Paraffin-embedded tissue blocks were cut with a rotary microtome into 4-µm serial sections and processed for immunohistochemical staining. Apoptotic cells were detected using the DeadEnd Colorimetric terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) system (Promega, Madison, WI). In other studies, sections were stained for p53 and PCNA as described (11). Microscopic immunohistochemical analyses were done using a Zeiss Axioscop 2 microscope (Carl Zeiss, Inc., Jena, Germany). Mean ± SE values were determined by evaluating multiple fields from each group.

Preparation of Tissue Homogenates and Western blotting

Epidermis, after removing the dermis from the skin by scraping, and tumors were homogenized in lysis buffer using a Polytron homogenizer and then centrifuged at 14,000 × g (11, 13). Supernatants were used to estimate protein concentrations, and 30 to 70 µg protein/sample was applied to a Tris-glycine gel; proteins resolved were transferred on to nitrocellulose membrane and blocked for 1 hour with 5% nonfat dry milk. Membranes were incubated with primary antibodies overnight at 4°C and then with appropriate secondary antibody followed by enhanced chemiluminescence detection. The antibodies used were E2F1, E2F2, E2F3, cyclin A, cyclin E, cyclin B1, cyclin D1, CDC2, CDC25C, CDK2, CDK4, and pRb110 (Santa Cruz Biotechnology, Santa Cruz, CA); p53 (Novocastra Laboratories Ltd., Newcastle upon Tyne, United Kingdom); phosphorylated and total Akt, extracellular signal-regulated kinase 1/2, c-Jun NH2-terminal kinase 1/2, and MAPK/p38, pCDC2, pRb(Ser780), cleaved poly (ADP-ribose) polymerase, and cleaved caspases-3 (Cell
Signaling Technologies, San Diego, CA); Cip1/p21 and Kip1/p27 (Lab Vision, Fremont, CA); pH2A.X(Ser139) (Upstate, Lake Placid, NY); and PCNA (DAKO, Glostrup, Denmark). Western immunoblots were scanned with Adobe Photoshop 6.0 (Adobe Systems, Inc., San Jose, CA), and the mean band density was analyzed by the Scion Image program (NIH, Bethesda, MD). Films were subjected to multiple exposures so that the band density was in the linear range.

Statistical Analysis

Data were analyzed using SigmaStat 2.03 software, and the significance between UVB alone versus all other groups was determined by one-way ANOVA followed by the Bonferroni t test for multiple comparisons. Between groups comparison was done by t test, with P < 0.05 considered to be statistically significant.

Results

Silibinin Up-regulates E2F1 and Decreases E2F2, E2F3, p53, p21, and p27 Proteins and Apoptosis in Skin Chronically Exposed to UVB

E2F1 is elevated and stabilized when cells or animals are exposed to DNA-damaging agents, such as UV (9, 14, 15). No reports show the effects of long-term chronic UVB exposure on E2F1 level in skin, an exposure that mimics those in humans that lead to nonmelanoma skin cancers. Contrary to previous reports with a single UVB exposure, chronic UVB exposure causes a decreased E2F1 level in skin epidermis (Fig. 2A). Topical silibinin treatment before or after UVB exposure or dietary silibinin feeding strongly increased the E2F1 level in both 15 and 25 weeks experiments (Fig. 2A). There is no report for in vivo effect of chronic UVB exposure on E2F2 and E2F3 levels that positively regulate cell cycle progression (16, 17). In contrast to the effect of chronic UVB exposure on E2F1, this treatment increased E2F2 and E2F3 in skin, but they decreased almost to normal skin epidermis levels by silibinin treatment (Fig. 2A).

Mice lacking E2F1 are sensitive to UVB-induced apoptosis in epidermis, but this is inhibited in transgenic mice overexpressing E2F1 (9). Because chronic UVB diminished the level of E2F1 in skin that was strongly reversed together with an increase following silibinin treatment, we analyzed skin samples for the markers of DNA damage and apoptosis. As shown in Fig. 2A, chronic UVB exposure resulted in a strong H2A.X(Ser139) phosphorylation and cleavage of caspase-3 and poly(ADP-ribose) polymerase at both 15 and 25 weeks of the study, and all these events were strongly inhibited by silibinin treatments in three different protocols (Fig. 2A). Together, these results with silibinin are in the favor of recent reports showing the role of enhanced E2F1 expression in the inhibition of apoptosis (9, 18). To further support this notion, quantitative apoptosis was also measured by TUNEL staining, where silibinin inhibited UVB-induced apoptosis in the epidermis by 65% to 72% and 49% to 59% (P < 0.001) in 15 and 25 weeks chronically UVB-exposed skin samples, respectively (Fig. 2C).

Depending on the extent of damage caused by DNA-damaging agents, including UVB, p53 is also well known to mediate cell cycle arrest for DNA repair and/or apoptosis. It has been observed that the level of p53 accumulation depends on the extent of DNA damage and that lower levels of p53 correspond to cell cycle arrest (19). p53 also transcriptionally activates Cip1/p21 for cell cycle arrest in response to UVB-induced DNA damage (11, 12, 20); however, it has been shown that E2F1 can inhibit the activity of p53 (21). Further, Cip1/p21 degradation is essential for the optimal DNA repair (22). Consistent with these reports and our observation that silibinin increases E2F1 level in chronically UVB-exposed mouse skin, it also decreased the protein levels of both p53 and Cip1/p21 in chronically UVB-exposed mouse skin (Fig. 2B). Similarly, we also observed a decrease in Kip1/p27 protein level by silibinin in these skin samples (Fig. 2B). A decrease in p53 was further confirmed by immunostaining, which showed 72% to 79% and 58% to 66% (P < 0.001) reduction in p53-positive cells in 15 and 25 weeks of skin samples from silibinin-treated and UVB-exposed groups compared with UVB alone, respectively (Fig. 2D). Taken together, these findings suggest that silibinin-caused increase in E2F1 possibly mediates suppression of p53 and CDK inhibitors to enhance DNA repair together with a decrease in apoptosis.

Silibinin Modulates G1 and G2-M Regulators in Skin Chronically Exposed to UVB

For the above suggestion that silibinin has a role in DNA repair and the fact that cell cycle arrest is an essential requirement for DNA repair (22), we expected that silibinin would alter the levels of CDKs and cyclins leading to a cell cycle arrest (23). Using UVB-induced skin tumors, we have reported recently that silibinin decreases the protein levels of CDKs and cyclins involved in G1 phase of the cell cycle (11); however, it is not yet known whether silibinin also modulates these cell cycle regulators in chronically UVB-exposed skin epidermis at the time of tumor appearance (15 weeks) and in uninvolved skin from tumor-bearing mice (25 weeks). In the present study, immunoblot analysis showed increased levels of CDK2, CDK4, cyclin A, cyclin E, and cyclin D1 in chronically UVB-exposed mouse skin compared with sham-irradiated control (normal) skin samples, and silibinin treatments in three different protocols strongly reversed these increases to the levels almost comparable with the normal skin (Fig. 3A). For the first time, we also observed that chronic UVB exposure causes a sustained level of phosphorylated pRb (p110) via pRB(Ser780) phosphorylation in skin, which is in accord with the increased levels of G1-phase CDKs and cyclins (Fig. 3A). In both sets of chronic UVB-exposed skin samples, consistent with the decreasing effect on CDKs and cyclins, silibinin also completely inhibited UVB-induced pRb(Ser780) phosphorylation (Fig. 3A). These findings suggest that silibinin possibly targets G1-phase CDKs and cyclins and downstream pRb to check G1-S transition needed for DNA repair.

Because G2-M arrest also mediates DNA repair, we next analyzed the effect of silibinin on CDC25C, CDC2, and
cyclin B1 levels, which ultimately control G2-M transition (24). Chronic UVB exposure increased the level of CDC25C phosphatase causing decreased phosphorylation of CDC2 and also increased total CDC2 and cyclin B1 levels compared with unexposed normal skin (Fig. 3B), suggesting a possible increased rate of cycling through G2-M transition. Silibinin treatments decreased total CDC25C, CDC2, and cyclin B1 protein levels (Fig. 3B). Furthermore, a decrease in CDC25C level following silibinin treatment was accompanied with a strong increase in phosphorylated CDC2 (Tyr15) (inactive) level, which was not detectable in control and chronic UVB-exposed samples (Fig. 3B). Taken together, these results suggest that silibinin induces both G1 and G2-M arrests for possible DNA repair following genotoxic effect of UVB in mouse skin.

**Silibinin Inhibits UVB-Induced Mitogenic and Survival Signaling in Chronically UVB-Exposed Skin**

UVB-caused activation of MAPKs and Akt modulates cell proliferation, survival/apoptosis, and cell cycle progression; these events collectively influence photocarcinogenesis (11, 25). In the present study, silibinin inhibited chronic UVB-induced phosphorylation of extracellular signal-regulated kinase 1/2, c-Jun NH2-terminal kinase 1/2, and p38 kinase as well as Akt in both 15- and 25-week skin samples, which were not due to the change in their total protein levels (Fig. 4A). Further, in immunoblotting (Fig. 4A) and immunohistochemical (Fig. 4B) analyses, silibinin treatments in three different protocols decreased UVB-induced PCNA levels in skin epidermis. The decrease in PCNA-positive cells was 76% to 79% to 56% to 58%
(\(P < 0.001\)) in 15- and 25-week skin samples compared with their UVB alone exposed samples, respectively (Fig. 4B). These results suggest that inhibitory effect of silibinin on UVB-induced mitogenic and survival signaling could be associated with decreased cell proliferation in mouse skin epidermis.

**Silibinin Inhibits UVB-Induced Skin Tumorigenesis**

The above observed inhibitory effects of silibinin on various molecules and biological events collectively suggest a chemopreventive effect of this phytochemical on UVB-induced skin tumorigenesis. Indeed, silibinin treatments delayed the appearance of the first tumor by 2 to 4 weeks (\(P < 0.01–0.001\)) compared with UVB alone group (Fig. 5A). Furthermore, in UVB alone group, all the mice developed tumors by 19 weeks; however, it took 22 to 23 weeks to get tumors in all mice in silibinin-treated groups (Fig. 5A). Silibinin treatments in three different protocols also reduced the number of tumors per mouse throughout the experiment and showed 47% to 58% (\(P < 0.001\)) decrease in tumor multiplicity compared with UVB alone group at the end of the experiment (Fig. 5B). Tumor volume per mouse was also decreased from 346 ± 76 mm\(^3\) in UVB alone group to 117 ± 21 mm\(^3\), 98 ± 20 mm\(^3\), and 133 ± 18 mm\(^3\) before silibinin or after silibinin topical treatment or dietary feeding, respectively, accounting for 61% to 72% decrease (\(P < 0.001\); Fig. 5C). None of the silibinin treatments caused any decrease in diet consumption (data not shown) or body weight gain (Fig. 5D) compared with control mice. These results convincingly indicate the protective effect of topically applied as well as dietary-fed silibinin against UVB-induced tumorigenesis in SKH-1 mouse skin without any toxicity.

**Unlike Chronically UVB-Exposed Skin, Silibinin Decreases E2F1 Levels in Skin Tumors without Any Effect on E2F2 and E2F3**

Because silibinin increases E2F1 but decreases UVB-induced E2F2 and E2F3 levels in chronically UVB-exposed skin samples, we also assessed their levels in chronically UVB exposure–induced skin tumors. Some very interesting observations were made in these studies, where unlike UVB-exposed skin samples, E2F1 levels were almost 3-fold higher in the skin tumors compared with age-matched normal skin (\(P < 0.001\)). Silibinin treatment in three different protocols decreased UVB-induced E2F1 levels in skin tumors, which were comparable with that in normal skin (Fig. 6A and B). With regard to E2F2 and E2F3 levels, compared with normal skin and consistent with the results in chronically UVB-exposed skin samples, skin tumors also showed an increase in their levels (\(P < 0.05\) for E2F2, \(t\) test); however, the increase was not statistically significant for E2F3 due to its variable levels in different tumor samples (Fig. 6A and B). Furthermore, similar to the observations in UVB-exposed skin, silibinin treatment in three different protocols decreased UVB-induced E2F2 and E2F3 levels in skin tumors, but these changes were not statistically significant due to variability in the immunoblot results (Fig. 6A and B). Taken together, skin tumor analyses clearly suggest that, unlike chronically UVB-exposed skin (Fig. 2A), silibinin decreases E2F1 level in skin tumors without any considerable effect on E2F2 and E2F3.

**Unlike Chronically UVB-Exposed Skin, Silibinin Induces Apoptosis and p53, Cip1/p21, and Kip1/p27 Levels in Skin Tumors**

In our recent study, we have shown that silibinin protects against photocarcinogenesis via modulation of cell cycle

![Figure 3](image-url)

**Figure 3.** Effect of silibinin on UVB-modulated levels of G\(_1\) and G\(_2\)-M cell cycle regulators in skin epidermis. Skin epidermal cell lysates used for the study in Fig. 1 were analyzed for G\(_1\) cell cycle regulators (CDKs, cyclins, and pRb and its phosphorylation; \(A\)) and G\(_2\)-M cell cycle regulators (Cdc25C, Cdc2 and its phosphorylation, and cyclin B1 levels; \(B\)) by SDS-PAGE and immunoblotting using specific antibodies. Membranes were stripped and reprobed for \(\beta\)-actin as loading control.
regulators, MAPKs and Akt signaling, in chronically UVB-exposed skin tumors in SKH-1 hairless mice (11). However, based on our central focus, in the present study, on E2F1 increase by silibinin to its inhibitory effect on apoptosis in chronically UVB-exposed skin, it was important to also analyze these biological and molecular events in skin tumors to further support an association between silibinin-caused alterations in E2F1 level and apoptosis. Herein, we used identical UVB and silibinin doses and treatment protocols to that in recently published study (11); however, the UVB exposure frequency was twice weekly compared with the earlier study where 5 times weekly exposure was used. Therefore, the cumulative UVB dose was two fifths of that used in the earlier studies (11, 26). Both immunoblotting and immunohistochemical analyses were done in the chronic UVB-exposed skin tumors with or without silibinin treatment, and the levels of apoptosis, p53, and PCNA were assayed. In contrast to its protective effect in chronic UVB exposed skin, a proapoptotic effect of silibinin was observed in tumors as evidenced by strong levels of cleaved caspase-3 and cleaved poly(ADP-ribose) polymerase in all the silibinin-treated groups of tumors compared with UVB alone (Fig. 6A). Interestingly, in contrast to its effect in chronically UVB-exposed skin, silibinin showed a strong increase in p53 protein level in tumors together with an increase in both Cip1/p21 and Kip1/p27 protein levels compared with UVB alone group (Fig. 6A). In terms of its effect on proliferation, consistent with the results in skin, silibinin showed a strong decrease in PCNA levels in tumors in all the groups compared with UVB alone group (Fig. 6A).

Consistent with immunoblotting results, TUNEL staining also showed proapoptotic effect of silibinin in tumors (Fig. 6C), where it caused 60% to 80% (P < 0.001) increase in TUNEL-positive cells compared with UVB alone group (Fig. 6C). Immunostaining of tumor samples also supported an increased level of p53 in silibinin-treated groups compared with UVB alone group, where quantitative analyses showed 70% to 90% (P < 0.001) increase in p53-positive cells by silibinin (Fig. 6C). Similarly, antiproliferative effect of silibinin in tumors were also supported by PCNA immunostaining where it showed 41% to 51% (P < 0.001) decrease in PCNA-positive cells compared with UVB alone group (Fig. 6C). These results clearly show proapoptotic and antiproliferative effects of silibinin in UVB-induced skin tumors as opposed to antiapoptotic but antiproliferative effects in chronically UVB-exposed mouse skin.

As silibinin inhibited UVB-induced cell proliferation in both skin and tumors but inhibited apoptosis only in skin while increased it in tumors, we also assessed its effect on MAPKs and Akt phosphorylation in tumor samples.
In contrast to chronically UVB-exposed skin, silibinin treatments further enhanced the phosphorylated extracellular signal-regulated kinase 1/2, c-Jun NH2-terminal kinase 1/2, and p38 kinase levels from that of UVB alone group of tumors or normal skin. Similar to UVB-exposed skin, however, silibinin treatments decreased phosphorylated Akt levels in tumors. These results suggest that activation of MAPKs and inhibition of Akt by silibinin could also have a proapoptotic role in UVB-induced skin tumors. Because all these results obtained in the present study are identical and consistent with our recently reported observations (11), they are not shown here.

**Discussion**

E2F transcription factors regulate the expression of genes involved in cell cycle progression, DNA repair, cell proliferation, and apoptosis (27). Although E2F1, E2F2, and E2F3 are generally known as activating E2Fs, a clear picture for their transcriptional activity, which modulates different biological responses, is not completely understood. According to a classic model, transcriptional activities of E2Fs are controlled by retinoblastoma family proteins, where different E2Fs and retinoblastoma family members associate with different gene promoters under different cellular conditions (28). The expression and activity of E2F proteins are tightly regulated transcriptionally and post-translationally as a function of proliferation and differentiation status of the cell (29). It has been assumed that tumor suppressor activity of E2F1 is related to its ability to promote apoptosis (30, 31), although other studies suggest that apoptosis induction is not the major mechanism by which E2F1 suppresses tumor development (18). Recently, E2F1 overexpression in mouse epidermis has been shown to promote DNA repair and suppress apoptosis in response to UVB (9). Consistent with this report, our results showed a strong increase in epidermal E2F1 in silibinin-treated groups compared with UVB alone group in the chronically UVB-exposed skin. This finding for the first time suggest the possible role of E2F1 in suppressing apoptosis and enhancing repair of UVB-induced DNA damage by silibinin, as observed in a recent study where similar silibinin treatments strongly reduced (71–85%) cyclobutane pyrimidine dimer–positive cells in UVB-exposed skin epidermis (12, 32). Consistent with an increase in E2F1 levels, silibinin strongly inhibited apoptosis in chronically (15 and 25 weeks) UVB-exposed skin, further supporting a possible link between E2F1 increase and antiapoptotic response. When these results in chronically UVB-exposed skin were compared with those in chronically UVB exposure–induced skin tumors, an

![Figure 5](image-url)
opposite observation was made where UVB increased E2F1 level compared with normal skin and silibinin treatments decreased the elevated E2F1 level in tumors to almost those in normal skin. Furthermore, unlike in chronically UVB-exposed skin where silibinin inhibited apoptosis, it was enhanced in skin tumors. These results convincingly show the differential effects of silibinin on E2F1 and associated apoptotic event in chronically UVB-exposed skin versus skin tumors in SKH-1 hairless mice, which might have an important role in overall efficacy of silibinin against UVB-caused skin damages and photocarcinogenesis.

The mechanism/s by which E2F1 could suppress apoptosis is not completely elucidated. Recently, it was reported that E2F1 could suppress UVB-induced apoptosis by inhibiting the activity of p53 and subsequently p53-dependent activation of Cip1/p21 (33). Usually, p53 induction is known for increased apoptosis and DNA repair (19); however, it can either stimulate or repress UV-induced apoptosis depending on the experimental conditions (33, 34). Consistent with these reports, silibinin decreased UVB-induced p53 and Cip1/p21 protein levels in skin; however, an absolutely opposite observation was again made in skin tumors. Taken together, these observations and those related to E2F1 and apoptosis clearly suggest a possible involvement of these mechanisms in an overall biological response. For example, silibinin-caused increase in E2F1 level relates with a decreased p53 and Cip1/p21 as well as apoptosis in skin, whereas silibinin-caused decrease in E2F1 level associates with an increase in p53 and Cip1/p21 as well as apoptosis in tumors. It is important to emphasize here that, although these associations are convincing, further studies are needed to establish

Figure 6. Effect of silibinin on E2F, apoptosis, p53, and cell proliferation in UVB-induced skin tumors. A, tumors harvested at the end of 25 wks of study in Fig. 4 were analyzed by immunoblot analysis as mentioned in Materials and Methods. B, densitometric values (arbitrary number) for E2F1 blots. Left, columns, SE; bars, mean of 5 normal skin samples and 10 tumor samples from the UVB or UVB and silibinin treatment groups. Similarly, the band intensity for E2F2 and E2F3 are from three normal skin samples and six tumor sample (left). * P < 0.001; ‡ P < 0.05 versus UVB group, t test. C, the tumors from the 25 wks of study were immunohistochemically analyzed for TUNEL, p53, and PCNA and quantified for percentage of positive cells for TUNEL, p53, and PCNA as mentioned in Materials and Methods. Columns, mean; bars, SE. * * P < 0.001 versus UVB group, Bonferroni t test. Not significant (NS) versus UVB alone group.
a causal role of one event in driving the other and vice versa and their biological significance in apoptosis. It would also be important to investigate in future how silibinin distinguishes between epidermal cells versus tumor cells for its differential effect on these tumor suppressors as well as apoptosis.

References

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