Abstract. Several environmental and genetic factors are involved in skin cancer induction, however exposure to chemical carcinogens and solar ultraviolet (UV) radiation are primarily responsible for several skin diseases including skin cancer. Chronic exposure of solar UV radiation to the skin leads to basal cell and squamous cell carcinoma, and melanoma. Chemoprevention of skin cancer by consumption of naturally occurring botanicals appears a practical approach and therefore world-wide interest is considerably increasing to use these botanicals. Sunscreens are useful but their protection is not ideal because of inadequate use, incomplete spectral protection and toxicity. Silymarin, a plant flavonoid isolated from the seeds of milk thistle (Silybum marianum), has been shown to have chemopreventive effects against chemical carcinogenesis as well as photocarcinogenesis in various animal tumor models. Topical treatment of silymarin inhibited 7,12-dimethylbenz(a)anthracene-initiated and several tumor promoters, like 12-O-tetradecanoylphorbol-13-acetate, mezerein, benzoyl peroxide and okadaic acid, induced skin carcinogenesis in mouse models. Similarly, silymarin also prevented UVB-induced skin carcinogenesis. Wide range of in vivo mechanistic studies indicated that silymarin possesses antioxidant, anti-inflammatory and immunomodulatory properties which may lead to the prevention of skin cancer in in vivo animal models. The available experimental information suggests that silymarin is a promising chemopreventive and pharmacologically safe agent which can be exploited or tested against skin cancer in human system. Moreover, silymarin may favorably supplement sunscreen protection and provide additional anti-photocarcinogenic protection.

1. Introduction

Silymarin is a plant derived flavonoid which is extracted from the fruits and seeds of the milk thistle (Silybum marianum L. Gaertn.) (1). Milk thistle belongs to the family of Asteraceae. Chemically, silymarin is a flavonolignan and consists of a mixture of mainly three flavonoids, silybin (silibinin), silydianin and silychristin (2,3). Silibinin is the major component (70-80%) found in silymarin and is thought to be the most biological active compound (Fig. 1). Pharmacological studies revealed that silymarin is non-toxic even at higher physiological doses, which suggests its safer use for the treatment of various diseases (4). Experimental evidence suggests that there is no significant difference between silymarin and silibinin in terms of chemopreventive or biological activities in several in vitro and in vivo animal models (5,6, and refs. therein). Silymarin has been used to treat disorders of the spleen, liver and gall bladder since the 4th century BC. Silymarin has been primarily used in liver disorders including hepatitis, alcoholic liver diseases and cirrhosis (4,7,8) and is also useful for toxin-induced liver toxicity, including poisoning from a fungus called death cap mushroom (Amanita phalloides) (9). Initially, our group has tested and determined the skin cancer chemopreventive effects of silymarin in animal system using chemical carcinogenesis and photocarcinogenesis mouse models (10-12). Since then extensive chemopreventive studies have been performed in several in vitro and in vivo animal models to test the efficacy of silymarin and establish the mechanism of action against skin carcinogenesis. This report highlights the chemopreventive effects and possible mechanism of action of silymarin or silibinin in vivo cell culture model and in vivo animal models of skin carcinogenesis.

2. Chemical carcinogenesis

Laboratory animals have been used as the convenient model to study in vivo mechanism of chemical carcinogenesis and...
Anti-chemical carcinogenic effect of silymarin.

Since tumor progression is an irreversible step and leads to the formation of benign tumors or controlling differentiation (13). The promotion stage is an irreversible step in which genetic change(s) occur in gene(s) 13-acetate (Fig. 2; TPA). Initiation stage is essentially an irreversible step which genetic change(s) occur in gene(s) controlling differentiation (13). The promotion stage is reversible and leads to the formation of benign tumors or papillomas. Tumor progression is an irreversible step and probably requires additional insult to the cellular genome of papillomas. Tumor progression is an irreversible step and probably requires additional insult to the cellular genome of papillomas. Tumor progression is an irreversible step and probably requires additional insult to the cellular genome of papillomas.

Figure 1. Chemical structure of silibinin, the major and most biological active component of silymarin.

Figure 2. Multistage chemical carcinogenesis showing initiation, promotion and progression stages.

modulation of sequential steps involved in this process. It is recognized that chemical carcinogenesis is a multifactorial, and a multistep process of at least three distinct stages: initiation, promotion and progression (13; Fig. 2). In murine skin chemical carcinogenesis, tumors are generally induced by a single topical application of the chemical carcinogen, termed initiating agents, such as 7,12-dimethylbenz(a)-anthracene (DMBA), followed by repeated application of the tumor promoting agent, such as 12-O-tetradecanoylphorbol-13-acetate (Fig. 2; TPA). Initiation stage is essentially an irreversible step which genetic change(s) occur in gene(s) controlling differentiation (13). The promotion stage is reversible and leads to the formation of benign tumors or papillomas. Tumor progression is an irreversible step and probably requires additional insult to the cellular genome of the papilloma (13-15).

**Anti-chemical carcinogenic effect of silymarin.** Since tumor promotion stage is reversible, this stage is most suitable to inhibit or control the process of carcinogenesis. Most of the antioxidants have been shown to inhibit the tumor promotion stage as oxidative stress has been shown to play an important role in tumor promotion (13,16). Since, silymarin possesses antioxidant properties and, tumor promoters induce oxidative stress during tumor promotion, we tested silymarin for its anti-carcinogenic effects in cancer-prone SENCAR mice. We showed that topical treatment of silymarin significantly inhibited TPA-induced ornithine decarboxylase activity (10). The enzyme ornithine decarboxylase has been used as a marker of tumor promotion. This observation suggested that silymarin might have anti-tumor promotion properties. Subsequently, we demonstrated that topical treatment of silymarin afforded significant chemopreventive effects in various chemical carcinogenesis models (12). Silymarin inhibits both stage I and stage II of tumor promotion in DMBA-TPA and DMBA-Mezerein SENCAR mouse skin carcinogenesis model, respectively (12). The chemopreventive effect of silymarin was observed in terms of tumor incidence (% mice with tumors), tumor multiplicity and tumor size throughout the experiment when compared with that of non-silymarin treated animals. Similarly, topical treatment of silymarin afforded significant protection against free radical generating tumor promoter, benzoyl peroxide, and another tumor promoter okadaic acid-caused tumor promotion in DMBA-initiated SENCAR mouse skin (17). Oral administration of silymarin also resulted in inhibition of tumor incidence, tumor multiplicity and tumor growth or size in DMBA-initiated and TPA promoted mice. It was also observed that established tumors were found to be regressed by the effect of silymarin treatment (5).

3. Mechanism of chemoprevention in chemical carcinogenesis

The mechanism of the anti-carcinogenic effect of silymarin was evaluated in vitro and in vivo animal models. Tumor promoters, specifically phorbol esters, have been shown to induce oxidative stress as one of the mechanism of tumor promotion. This oxidative stress is generally induced because of tumor promoter-induced infiltration of leukocytes. Infiltrating leukocytes have been shown to be a major source of reactive oxygen species generation. Oxidative stress leads to the damage of macromolecules such as DNA, lipids and proteins (13). Silymarin inhibits TPA-caused depletion of antioxidant enzyme activities, such enzymes are superoxide dismutase, catalase and glutathione peroxidase, in the epidermis (18), which results in reduction of oxidative stress. Silymarin inhibits TPA-caused lipid peroxidation in mouse skin, thus supporting its in vivo antioxidant activity (12). The inhibition of oxidative stress by silymarin can shift the equilibrium of carcinogen metabolism, gene expression and enzyme activity, and thus prevent the process of skin carcinogenesis. Edema and hyperplastic responses are considered as inflammatory markers, and play a crucial role in skin tumor promotion (13). Application of tumor promoters, e.g. TPA, to mouse skin induces the development of edema and hyperplastic response in epidermal keratinocytes. Treatment of silymarin resulted in inhibition of TPA-induced edema, hyperplastic response and myeloperoxidase activity in the skin (18). Myeloperoxidase is commonly used as a marker of tissue infiltration. Silymarin reduced TPA-induced induction of epidermal lipoxygenase, interleukin-1α and cyclooxygenase-2 expression but not cyclooxygenase-1 activity (18). Thus, the antioxidant and anti-inflammatory effect of silymarin may be the possible mechanism of prevention of skin cancer in chemical carcinogen-induced skin carcinogenesis models. Inflammatory cytokines such as TNFα and IL-1α have been shown to be involved in tumor promotion (19,20). It has been shown that silymarin inhibits
TPA- and okadaic acid-induced expression of TNFα mRNA in a dose-dependent manner in SENCAR mouse skin (21). Silymarin treatment to mouse skin inhibits TPA-induced expression of IL-1α mRNA and IL-1α protein as well (18). Some in vitro studies also have been conducted in different cell culture model to demonstrate the anti-carcinogenic effect and possible mechanism of action of silymarin or silibinin. Treatment of silybin to human epidermoid carcinoma A431 cells was found to result in a significant decrease in MAPK activation (5). This in vitro study suggested a possible underlying molecular mechanism involved in inhibition of proliferation and induction of apoptosis in human epidermoid cancer cells. In some in vitro studies silymarin/silibinin has been shown to control cell cycle regulators or check points in skin cancer cells through which cells undergo apoptosis (22). These in vitro and in vivo observations provide ample evidence that anti-carcinogenic effect of silymarin/silibinin against chemical carcinogenesis is mediated through various mechanistic pathways including its anti-inflammatory and antioxidant properties.

4. Photocarcinogenesis

**Solar ultraviolet (UV) radiation and skin.** Changes in life style over the past several decades have provided individuals with much greater amounts of time for recreational activities and much of this time has been spent outdoors. As a result there has been alarming increase in the incidence of solar UV radiation induced skin disorders including skin cancers. Acute UV overexposure causes sunburn, more chronic exposure of UV leads to basal cell and squamous cell carcinoma, melanoma, photoaging and immune suppression (23-26). Statistical analysis reveals that approximately 1.3 million new cases of non-melanoma skin cancer are diagnosed every year in the United States alone, thus solar UV radiation is considered as the most prevalent environmental carcinogen (27,28). The incidence of skin cancer has been increasing dramatically, and this increase is expected to continue as the population ages and larger amounts of UV radiation reach the surface of the Earth because of depletion of the ozone layer (23-25). According to the current projections, one in five Americans will develop at least one non-melanoma skin cancer during their lifetime. UVB (290-320 nm) component of the solar UV spectrum can act as tumor initiator, tumor promoter and as a complete carcinogen by damaging cellular macromolecules such as DNA, proteins and lipids (11,23,26). It is well documented that UV irradiation to skin induced the generation of reactive oxygen species (ROS), such as singlet oxygen, peroxyl radicals, superoxide anion and hydroxyl radicals which creates a state of oxidative stress in the target cells. Oxidative stress results when the formation of ROS exceeds the antioxidant defense ability of the target cells. ROS can also act as tumor initiator and tumor promoter by damaging cellular macromolecules, and by activating cell-signaling molecules (29-32). Thus oxidative stress or ROS has been implicated in many disease processes including skin cancer (26). It has been recognized that UV-induced DNA damage, predominantly in the form of cyclobutane pyrimidine dimers (CPD), plays an important role in immune suppression and skin cancer initiation. Accumulation of damaged DNA lesions results in mutations in critical genes and contributes to the development of non-melanoma skin cancers. Mammalian cells have efficient mechanisms to preserve genomic stability however, if the damage is too severe after a high dose of UV irradiation, cells have a mechanism to trigger apoptosis or programmed cell death to prevent the propagation of the damaged DNA (33,34). Thus CPD are implicated in UV-induced suppression of immune system (35,36) and carcinogenesis (36,37), and there is a close association between UV-induced immune suppression and carcinogenesis (38,39). The DNA strand breakage, thymine glycols and 8-hydroxyguanine are all forms of oxidative DNA damage. Strand breakage of DNA is induced by both UVB (290-320 nm) and to a greater extent by UVA (320-400 nm) radiation (40).

**Botanicals and skin photoprotection.** The use of botanicals is receiving considerable interest to protect skin from the adverse biological effects of solar UV radiation. These botanical supplements hold promise to be used as a complementary and alternative medicine for various skin disorders. The use of botanicals in skin care products has received attention by researchers, industry, consumers and the news media particularly for the protection of human skin from the damaging effects of external environmental stimuli, such as solar UV radiation. The botanicals which possess antioxidant and anti-inflammatory properties and immunomodulatory effects are very important in terms of skin photoprotective effects. Several studies have demonstrated the efficacy of naturally-occurring botanicals in animal models related to protection against UV radiation-induced inflammation, immunosuppression, oxidative stress and/or cancer. These botanicals are vitamin E (41), green tea polyphenols (42,43), garlic (44), ginger (45), silymarin (11,46), vitamin C (41), all-trans retinoic acid (47), proanthocyanidins from grape seeds (48) and lutein (carotenoids) (49) etc. Since oxidants play an important role in several skin disorders including the initiation and promotion stages of multistage skin carcinogenesis, the antioxidants can be targeted for intervention at the initiation, promotion or progression stages of multistage skin carcinogenesis or other age-related skin disorders such as premature aging of the skin (14,50,51). It has been shown that some dietary components or environmental mutagens and carcinogens, including solar UV radiation to which humans are constantly exposed, exert their adverse biological effects, at least in part, via the generation of ROS. ROS play a major role in the induction of cancer, specifically, at the promotion stage of carcinogenesis (52,53). Intake of botanical anti-oxidants, therefore, has been suggested as an important preventive strategy against the toxic effects of mutagenic and carcinogenic agents (52-55). We and others have demonstrated the photoprotective effects of polyphenols from green tea (50,54), silymarin from milk thistle (11,12,46) and proanthocyanidins from grape seeds against chemical tumor promoters as well as UV radiation-induced skin tumor initiation and promotion in animal models (48). The present review specifically deals with the skin photoprotective effect of silymarin, a plant flavonoid, which has been shown to have antioxidant and immunomodulatory effects.
Anti-inflammatory effects of silymarin. Clinical observations and experimental evidence have long documented an association between chronic inflammation and increased incidence of tumor formation occurring at the inflammatory site (65). The mediating agent(s) between inflammation and the development of the tumor at the inflammatory site has been proposed to be oxidative products produced by the inflammatory leukocytes (43,50,66). We observed that UVB exposure to both mouse and human skin induces infiltration of leukocytes which are the major source of oxidative stress such as H₂O₂ and nitric oxide production (14,43,67). Topical treatment of silymarin inhibited UVB-induced edema and hyperplastic response in SKH-1 hairless mice when compared with that of non-silymarin treated but UVB exposed mice (11). Edema was measured in terms of increase in bi-fold skin thickness and ear punch weight. UV induced infiltration begins significantly after 24 h of UV irradiation and persists up to 72 h when compared with the non-UV irradiated control mice. Treatment of silymarin significantly inhibited UV-induced infiltration of leukocytes which further supports the anti-inflammatory effect of this botanical agent. Myeloperoxidase is commonly used as a marker of tissue infiltration. The increase in myeloperoxidase activity after UVB exposure indicates an influx of leukocytes to the inflamed skin. Topical treatment of silymarin before UV irradiation resulted in significant reduction in myeloperoxidase activity both in epidermis and dermis (46). Reduction in myeloperoxidase activity by silymarin suggests the inhibition of UV-induced infiltration of inflammatory leukocytes, and thus anti-inflammatory effect of silymarin in this model.

Antioxidant effect of silymarin. Skin is easily accessible and constantly exposed to free radical-generating agents such as solar UV radiation, ozone and other environmental pollutants (26). Studies have shown the involvement of oxidative stress in skin carcinogenesis (26), and also demonstrated that a sophisticated enzymatic and non-enzymatic antioxidant defense system including catalase, superoxide dismutase and glutathione peroxidase counteracts and regulates overall ROS levels to maintain physiological homeostasis (Fig. 3). Increased ROS level is detrimental to target cells. We have shown that UV irradiation of SKH-1 hairless mice results in a significant depletion of antioxidant enzymes in the skin (68,69). Topical treatment of silymarin to SKH-1 hairless mice resulted in inhibition of UVB-induced intracellular production of H₂O₂ in both epidermis and dermis when analyzed by immunohistochemistry and biochemical analytical procedures and compared...
with non-silymarin treated animals (46). It has been noted that UVB-induced oxidative stress was significantly inhibited through the inhibition of UV-induced infiltration of leukocytes by silymarin treatment. UVB irradiation to skin also induced the expression of inducible nitric oxide synthase and resulted in increased amount of nitric oxide production compared to that of non-UVB irradiated control mice. Treatment of silymarin to mouse skin affords significant protection against UVB-induced expression of inducible nitric oxide synthase and subsequently nitric oxide production (46). Further, silymarin also inhibited UVB-induced expression of cyclooxygenase-2 and its prostaglandin metabolites, such as PGE₂, PGF₂α, and PGD₂, which have been implicated in tumor promotion (11,26). The above information indicated the antioxidant nature of silymarin which have a role in photo-protection of oxidative stress-associated skin disorders including skin cancer.

Immunomodulatory effects of silymarin. UV radiation has multiple effects on the immune system (36,38,39). There is ample clinical and experimental evidence to suggest that immune factors contribute to the pathogenesis of solar UV light-induced skin cancer in mice and probably in humans as well (20,26). Chronically-immunosuppressed patients living in regions of intense sun exposure experience an exceptionally high rate of skin cancer (70). This observation is consistent with the hypothesis that immune surveillance is an important mechanism designed to prevent the generation and maintenance of neoplastic cells (71). Further, the incidence of skin cancers, especially squamous cell carcinomas (SCCs), is also increased among organ transplant recipients (72-75). These studies provide evidence in support of the concept that immune suppression promotes the risk of skin cancer development.

UVB radiation-induced DNA photoproducts and reactive oxygen intermediates have been implicated as damaging to cutaneous immunity (26,36). It has also been suggested that the release of cytokines following UVB radiation plays a significant role in UVB-induced immunosuppression and, thus, may be an important factor in the growth and development of immunogenic UV-induced skin tumors (26). Also of potential significance is the fact that a number of tumors, including melanoma and non-melanoma skin cancer, appear to produce IL-10 (76-78). The immunosuppressive effects of IL-10 may be one of the mechanism by which these tumors escape immunologic control (78). Studies associated with silymarin in an animal model have indicated that topical treatment of silymarin to mouse skin prevents UVB-induced suppression of contact hypersensitivity (CHS) response to contact sensitizer dinitrofluorobenzene (46). The reversal of UVB-induced immunosuppression by silymarin treatment was associated with decreased production of IL-10 in UV irradiated skin and draining lymph nodes (46). A number of in vitro studies have shown that IL-10 inhibits antigen presentation (79,80) and secretion of cytokines by macrophages (81,82) thereby down-regulating CHS responses. Some in vivo studies have also shown the effects of IL-10 in T-cell-mediated reactions. Intraperitoneal administration of IL-10 to mice inhibits delayed type hypersensitivity responses (83). Intraperitoneal treatment of anti-IL-10 antibody to mice prevented UVB-induced tolerance induction (84). Thus inhibition of IL-10 production by silymarin treatment appears to prevent UVB-induced immune suppression in mice (46). The number of IL-10 producing cells in UV exposed epidermis was increased in comparison to epidermis not exposed to UV (46). Treatment of silymarin significantly reduces the number of IL-10 producing cells and its production in UV irradiating skin, which is accompanied by a reduction in infiltrating leukocytes.

It has been demonstrated that UV-induced infiltrating cells, particularly MHC⁺ CD11b⁺ cells, have a role in UVB-induced suppression of CHS (85). It was observed that prevention of UV-induced suppression of CHS by silymarin was associated with the reduction in MHC⁺ CD11b⁺ cell type into UVB-irradiated skin. This is also confirmed by the fact

Figure 3. Antioxidant defense mechanism in the skin. Target sites of silymarin are shown by blocking heads. These target sites may be responsible for reduction of UV radiation-induced generation of reactive oxygen species in the skin after silymarin treatment.
that silymarin treatment inhibited myeloperoxidase activity, a marker of tissue infiltration, in UVB-irradiated skin (46). Hammerberg et al (85,86) demonstrated that blocking of infiltrating leukocytes using anti-CD11b antibody or treatment with soluble complement receptor type-I blocked UV-induced immune suppression and tolerance induction in C3H/HeN mice. Thus, it appears that prevention of UV-induced suppression of CHS by silymarin is mediated through the suppression of infiltration of MHC+CD11b+ cell type. Such type of immunological modulation in UV-irradiated skin was also found with the topical treatment of green tea polyphenols (87). Therefore, it is evident that natural botanicals possess the capability of modulating UV-induced immunological responses in in vivo systems.

Photochemical damage to DNA, predominantly in the form of CPD, is one of the major effects of UV radiation. It has been demonstrated that CPD plays a crucial role in UV-induced immune suppression and carcinogenesis (35-37). Employing immunohistochemical techniques, recently it has been shown that topical application of silibinin prevents UV-induced DNA damage in vivo mouse skin when determined in the form of CPD formation. Additionally in the same in vivo animal system, silibinin treatment resulted in up-regulation of p53. One of the major effects of p53 is to initiate apoptosis in cells that have sustained significant DNA damage (88). Thus, prevention of CPD formation and augmentation of p53 by silibinin in UV-exposed skin may represent one of the possible mechanisms of prevention of cutaneous immune suppression and prevention of photocarcinogenesis as well.

6. Future prospects of silymarin for human use

Current experimental observations indicate that silymarin/silibinin possesses anti-inflammatory, antioxidant and anti-carcinogenic properties. This includes photoprotection against the sunburn response, DNA damage, non-melanoma skin cancer and immune suppression which show promise that silymarin can complement and enhance the photoprotective effect of currently available sunscreens. It is important because no sunscreen provides full spectral protection against UV light. Supplementation of skin care products or sunscreens with silymarin is likely to lead to further improvement in the photoprotective efficacy in human system. However, further studies are required in human system to determine cellular uptake, distribution and long-term effect of silymarin in the skin for their optimal photoprotection. More importantly, efforts are also required to develop more active derivatives of silymarin or silibinin which should have hydrophilic characteristics.

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References