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Short Communication

Silymarin inhibits melanin synthesis in melanocyte cells

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Abstract

Objectives The aim was to search for inhibitors of melanogenesis from natural resources.

Methods The inhibitory effect of silymarin on melanogenesis in a spontaneously immortalized mouse melanocyte cell line, Mel-Ab, was studied.

Key findings Silymarin significantly prevented melanin production in a dose-dependent manner with an IC50 value (concentration producing 50% maximal inhibition) of 28.2 μg/ml, without effects on cell viability. Also, silymarin inhibited L-DOPA oxidation activity of tyrosinase, the rate-limiting melanogenic enzyme, in cell based-systems but it did not directly affect cell-free tyrosinase activity. Furthermore, Western blot analysis indicated that silymarin decreased the expression of tyrosinase protein.

Conclusions This study suggests that the depigmenting effect of silymarin might be attributable to inhibition of tyrosinase expression and that silymarin may be useful as a natural skin-lightening agent.

Keywords melanogenesis; silymarin; skin-lightening; tyrosinase activity

Introduction

Melanins are synthesized within melanosomes of mammalian melanocytes. Although melanins protect the skin from harmful environments and sunlight, increased production of melanin can induce skin disorders, including acquired hyperpigmentation, such as melasma, post-inflammatory melanoderma and solar lentigo.[1] Skin lighteners are applied for the prevention and treatment of melasma, freckles and age spots in Western countries.[2]

Pigmentation can be regulated during melanocyte development and the major determinant of pigment phenotype is the melanocortin-1 receptor (MC1R).[3] In the mammalian epidermis, alpha-melanocyte-stimulating hormone (α-MSH), an agonist of MC1R, is produced and activates adenylyl cyclase to produce cyclic AMP (cAMP). The cAMP exerts its effect in part through protein kinase A (PKA). PKA, in turn, phosphorylates and activates cAMP-response element binding protein (CREB) that binds to cAMP-response element (CRE) present in the M promoter of the microphthalmia-associated transcription factor (Mitf) gene that is pivotal to the expression of melanogenic enzymes, including tyrosinase, tyrosinase-related protein-1 (TRP-1) and TRP-2.

Tyrosinase is a major melanosomal enzyme in melanogenesis, and catalyses the rate-limiting reaction of the melanogenic process.[4–6] Therefore, melanin production is mainly dependent on the expression and activation of tyrosinase.[7]

Silymarin is a polyphenolic flavonoid complex isolated from milk thistle (*Silybum marianum* L. Gaertn.), and consists of several flavonolignans, including silybin, isosilybin, silydianin, silychristin and other phenolic compounds.[8] Silymarin is a hepatoprotective drug that is widely used in the therapy of various liver diseases.[9] Moreover, the antioxidative, anticarcinogenic and anti-inflammatory effects of silymarin have been previously reported.[10,11] In particular, silymarin and silybin have been shown to prevent skin cancer;[12–14] however, an inhibitory effect of silymarin on melanogenesis has not yet been reported.

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In this study, we investigated the effect of silymarin on melanin synthesis and tyrosinase activity in a spontaneously immortalized mouse melanocyte cell line, Mel-Ab.

Materials and Methods

Reagents

Silymarin was purchased from Sigma-Aldrich Co. (St Louis, MO, USA). Silymarin was dissolved in dimethyl sulfoxide (DMSO) and stored at −20°C as a stock solution (100 mM). Cholera toxin, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), L-DOPA and mushroom tyrosinase were obtained from Sigma-Aldrich Co. Antibodies against tyrosinase (C-19) and actin (I-19) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, USA).

Cell cultures

The Mel-Ab is a mouse-derived spontaneously immortalized melanocyte cell line that produces large amounts of melanin.[15] Mel-Ab cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 nM TPA, 1 nM cholera toxin, 50 μg/ml streptomycin, and 50 U/ml penicillin at 37°C in a humidified incubator with 5% CO_2.

Cell viability assay

Cell viability was determined using a crystal violet assay.[15] Mel-Ab cells were seeded at a density of 1×10^5 cells per well of 6-well plates and incubated at 37°C. After incubating with test substance for 24 h, the culture medium was removed, and the cells were stained with 0.1% crystal violet in 10% ethanol for 5 min at room temperature and rinsed four times with distilled water. The crystal violet retained by adherent cells was then extracted with 95% ethanol. Absorbance was determined at 590 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Measurement of melanin content

Melanin content was measured as previously described[16] with slight modification. Mel-Ab cells were incubated at a density of 1×10^5 cells per well of 6-well plates at 37°C. After 24 h, the cells were treated with test substances in DMEM containing 2% FBS for four days. Cell pellets were dissolved in 1 ml of 1 M NaOH at 100°C for 30 min and centrifuged at 16 000*g* for 20 min. The optical density (OD) of the supernatants was measured at 400 nm using a microplate reader.

Tyrosinase activity

Tyrosinase activity was determined as previously described[17] with slight modification. Briefly, Mel-Ab cells were treated with test substances at various concentrations for four days, the cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed with phosphate buffer (pH 6.8) containing 1% Triton X-100. The cells were then disrupted by freezing and thawing, and the lysates were clarified by centrifugation at 10 000*g* for 5 min. After quantifying the protein levels and adjusting concentrations with lysis buffer, 90 μl of each lysate, containing equivalent amounts of protein, was placed in each well of a 96-well plate, and 10 μl of 10 mM L-DOPA was then added to each well. Control wells contained 90 μl of lysis buffer and 10 μl of 10 mM L-DOPA. After incubation at 37°C, the absorbance was measured every 10 min for at least 1 h at 475 nm using a microplate reader.

A cell-free assay system was used to test for direct effects on tyrosinase activity. Briefly, 170 μl of phosphate buffer containing various concentrations of test substances was mixed with 10 μl of mushroom tyrosinase (2000 U/ml) or with 10 μl of mouse tyrosinase containing 20 μg of total protein extracted from mouse melanocytes, and 20 μl of 10 mM L-DOPA was added to each well. Following incubation at 37°C, the absorbance was measured at 475 nm.

Western blot analysis

Mel-Ab cells were seeded at a density of 1×10^5 cells per well of 6-well plates and incubated at 37°C. After incubating with test substances for four days, the cells were harvested and lysed in cell lysis buffer (62.5 mM Tris-HCl, 2% sodium dodecyl sulfate (SDS), 5% β-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride, protease inhibitors (Complete; Roche, Mannheim, Germany), 1 mM Na_3VO_4, 50 mM NaF and 10 mM EDTA, pH 6.8). Twenty micrograms of protein per lane was separated by SDS-polyacrylamide gel electrophoresis and then transferred onto a PVDF membrane. The membrane was blocked with 5% non-fat milk in TBST buffer (25 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, pH 7.5) for 2 h at room temperature and subsequently incubated overnight at 4°C with anti-tyrosinase antibody at a dilution of 1 : 500. After washing with TBST buffer, the blot was incubated with horseradish peroxidase-conjugated anti-goat IgG antibody at a dilution of 1 : 1000 for 2 h at room temperature. The blotted antibody was visualized using a chemiluminescence method (ECL kit; Amersham, UK).

Statistics

All experimental data are presented as means ± SD. Differences between results were assessed using Kruskal–Wallis test followed by Dunn's test. $P < 0.05$ was regarded as significant.

Results

Effect of silymarin on melanogenesis in melanocytes

To investigate any possible cytotoxic effect of silymarin on Mel-Ab melanocytes, we treated the cells with silymarin at various concentrations and cell viability was determined by crystal violet assay. Silymarin did not show any significant cytotoxic effect on Mel-Ab cells within a concentration range of 1–50 μg/ml, indicating that the inhibitory effect of silymarin on melanin production was not attributable to its nonspecific cell toxicity (data not shown).

We next examined the effect of silymarin on melanogenesis in Mel-Ab melanocytes. The cells were exposed to 1–50 μg/ml silymarin for four days and their melanin content was measured. Silymarin had a dose-dependent inhibitory effect with 38.5%, 46.9% and 64.9% inhibition at 10, 20 and

50 μg/ml, respectively (IC50 28.2 μg/ml) (Figure 1). Phenylthiourea, a well-known inhibitor of tyrosinase and melanin synthesis, also showed an inhibitory effect with 79% inhibition at 10 μg/ml.[18]

Effect of silymarin on tyrosinase activity

Tyrosinase is a key enzyme in melanin synthesis. Thus, we next determined whether silymarin affected tyrosinase activity. To examine the cell-based tyrosinase inhibitory activity of silymarin, we treated Mel-Ab cells with silymarin for four days and then evaluated the tyrosinase activity of the cell lysates by measuring L-DOPA oxidation activity. As shown in Figure 2, tyrosinase activity was reduced dose-dependently in silymarin-treated cells with 21.9%, 33.8% and 55.5% inhibition at 5, 10 and 20 μg/ml. This is in good agreement with the inhibitory effects of silymarin on melanin synthesis (Figure 1).

Many skin-whitening agents inhibit the catalytic activity of mushroom tyrosinase. Thus, to test whether silymarin directly inhibited the tyrosinase enzyme, its effect in a cell-free system was investigated. However, as shown in Figure 3, silymarin did not inhibit mushroom tyrosinase activity even at 100 μg/ml, and a similar result was obtained using mouse tyrosinase. Kojic acid, a well-known tyrosinase inhibitor derived from fungal species, exerted a strong inhibitory effect on mouse and mushroom tyrosinase with 66.7% inhibition at 10 μg/ml.[19]

Effect of silymarin on expression of tyrosinase

To investigate the mechanism of depigmenting action shown by silymarin, western blot analysis was carried out with lysates of Mel-Ab cells. As shown in Figure 4, silymarin reduced the tyrosinase protein levels with a similar potency to its inhibitory activity on melanin synthesis and tyrosinase activity, which suggests that silymarin inhibits melanin synthesis by reducing tyrosinase protein expression in Mel-Ab melanocyte cells.

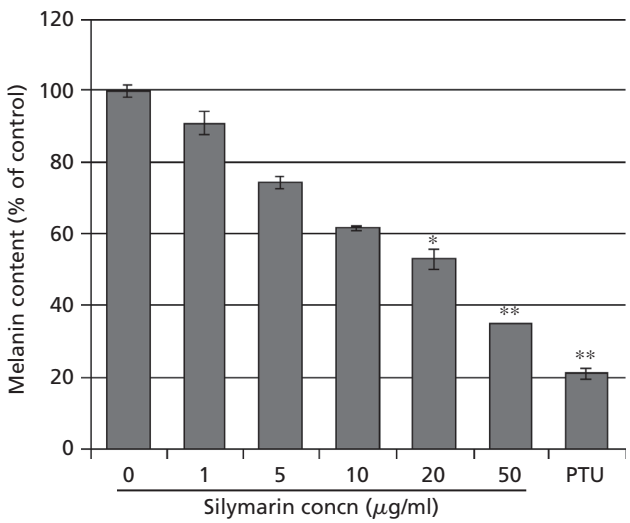

Figure 1 Effect of silymarin on melanin synthesis in Mel-Ab cells. Cells were cultured with 0–50 μg/ml of silymarin for four days and melanin content was measured as described in Materials and Methods. PTU, phenylthiourea. The results shown are the averages of triplicate experiments ± SD. *$P < 0.05$, **$P < 0.01$ vs control.

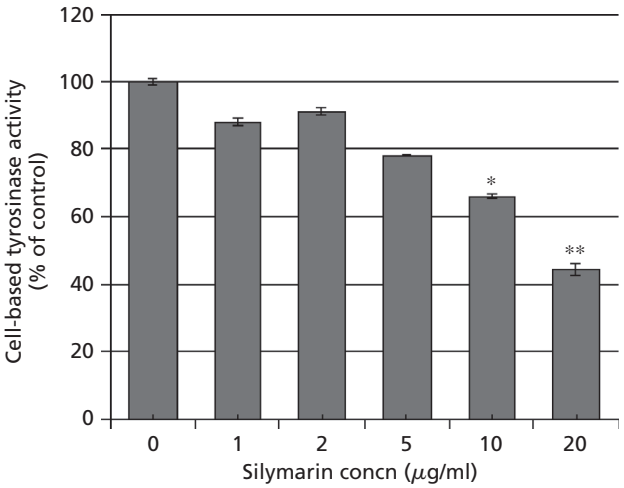

Figure 2 Effect of silymarin on tyrosinase activity in Mel-Ab cells. Cells were cultured with 0–20 μg/ml of silymarin for four days and cell-based tyrosinase activity was measured as described in Materials and Methods. The results shown are the averages of triplicate experiments ± SD. *$P < 0.05$, **$P < 0.01$ vs control.

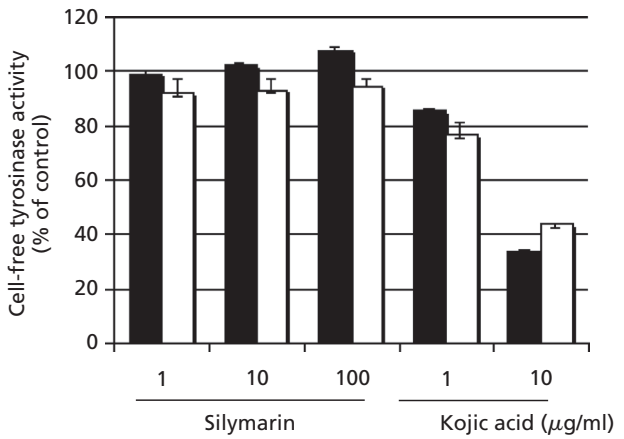

Figure 3 Effect of silymarin on cell-free tyrosinase activity. To test the direct effect of silymarin on mushroom and mouse tyrosinase, L-DOPA oxidase activity was measured as described in Materials and Methods. Shaded columns, mushroom tyrosinase; open columns, mouse tyrosinase. The results shown are the averages of triplicate experiments ± SD.

Discussion

Silymarin showed dose-dependent inhibitory effects on melanogenesis in Mel-Ab melanocytes without having any significant cytotoxic effects. Silymarin is widely used in human therapy to promote improved liver function because of its hepatoprotective and antioxidative properties.[20] Especially, silymarin and its major compound, silybin, have been reported to prevent skin cancer by modulation of cell cycle regulators, activation of mitogen activated protein kinase (MAPK)

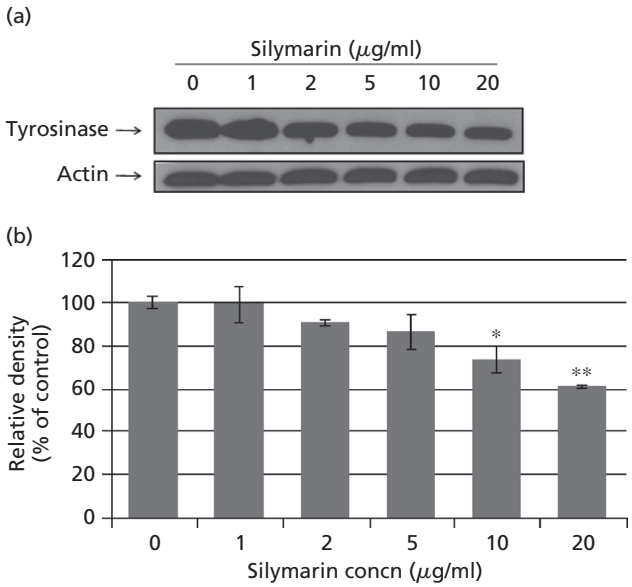

Figure 4 Effect of silymarin on the expression of melanogenic protein tyrosinase. (a) Mel-Ab cells were cultured with 0–20 μg/ml of silymarin for four days. Whole cell lysates were then subjected to western-blot analysis using antibody against tyrosinase. Similar results were observed in three independent experiments. (b) The relative density of the tyrosinase protein band is expressed as a percentage of the signal intensity relative to vehicle-treated control. The results shown are the averages of triplicate experiments ± SD. $*P < 0.05$, $**P < 0.01$ vs control.

pathways, and inhibition of Akt signalling.[12] Also, it is well known that silymarin exhibits anticarcinogenic and anti-inflammatory effects.[21–24] Some studies have also suggested that anti-inflammatory compounds could be useful for the prevention or treatment of post-inflammatory hyperpigmentation.[25] It has been reported that glabridin from licorice extracts,[26] ethanolic extracts from *Areca catechu* L[27] and globulusin A and eucaglobulin from *Eucalyptus globules*[28] show anti-inflammatory activity, as well as an anti-melanogenesis effect, by suppression of pro-inflammatory cytokines. It was also reported that silymarin suppressed the production of interleukin-1β (IL-1β) and prostaglandin E2 (PGE-2) produced by cyclooxygenase-2 (COX-2)[22] and also tumour necrosis factor-α (TNF-α).[24] In addition, silibinin, the major component of silymarin, decreased inducible nitric oxide synthase (iNOS) and COX-2, as well as nuclear factor κB (NF-κB).[23] Thus, it would appear that the anti-melanogenesis activity of silymarin might be related to its anti-inflammatory effect, but this link is yet to be characterized.

Tyrosine is a key enzyme in melanin synthesis and we found that silymarin dose-dependently reduced tyrosine activity in Mel-Ab cells but did not inhibit mushroom tyrosine activity in a cell-free system. This suggests that the inhibitory effect of silymarin on melanogenesis is not due to its direct inhibition of tyrosinase activity.

Controversial results were obtained when purified mushroom tyrosinase or cell lysates were employed.[25] For example, terrein, a fungal metabolite isolated from *Aspergillus terreus*, potently inhibited melanin synthesis but did not inhibit the catalytic activity of tyrosinase from mushroom or humans.[29] Recently, Sato *et al.*[30] reported that acetylsalicylic acid inhibited melanin synthesis by down-regulation of tyrosinase expression, but did not affect mushroom tyrosinase activity. In the same manner, silymarin decreased cell-based tyrosinase activity without direct inhibition of catalytic activity of this enzyme in a cell-free system.

Our results suggested that silymarin inhibits melanin synthesis by reducing tyrosinase protein expression in Mel-Ab melanocyte cells with a similar potency to which it inhibits melanin synthesis and tyrosinase activity. The proliferation and differentiation of mammalian epidermal melanocytes are regulated by numerous genes[31] and the surrounding tissue environment, especially keratinocytes. Moreover, it was reported that pigmentation could be regulated by keratinocyte-derived factors through receptor-mediated signalling pathways. Among these factors, PGE-2 and PGF-2α are known to be produced and released from keratinocytes, bind to EP1, EP3 and FP receptors, and stimulate melanogenesis through cAMP-independent phospholipase C, whereas α-MSH acts through the cAMP-mediated PKA pathway. Previous reports have examined the effects of prostaglandins on the melanogenic process. Abdel-Malek *et al.*[32] showed that PGE-1 and PGE-2 increased tyrosinase activity and Scott *et al.*[33] suggested that PGF-2α promoted pigmentation following ultraviolet radiation and during inflammation. Also, Sasaki *et al.*[34] reported stimulatory effects of PGF-2α and PGE-2 on follicular melanogenesis in a murine model. It was also reported that silymarin suppressed the production of PGE-2 in macrophages.[22] Thus, we assumed that the inhibition of tyrosinase expression by silymarin may be due to the suppression of PGE-2 production in this assay system. Further studies on the depigmenting mechanism of silymarin are now underway.

Conclusions

We evaluated the hypopigmentary effect of silymarin in Mel-Ab mouse melanocyte cells. Silymarin did not affect cell viability at the concentrations used, indicating that silymarin was not cytotoxic to Mel-Ab cells. Silymarin significantly reduced melanin contents with an IC50 value of 28.2 μg/ml and silymarin-induced hypopigmentation was found to be correlated with inhibition of cell-based tyrosinase activity but not catalytic activity of this enzyme. In addition, silymarin also reduced the tyrosinase protein level. These results suggest that silymarin inhibited melanin synthesis by reducing tyrosinase activity and expression. Further studies on the signalling pathways regulating tyrosinase are under investigation.

In summary, silymarin inhibited melanin synthesis by reducing the protein expression of tyrosinase without cytotoxicity in a mouse melanocyte cell line, Mel-Ab. Therefore, silymarin might have potential as a skin-lightening agent.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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