Astragalin Attenuates UVB Radiation-induced Actinic Keratosis Formation

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Abstract: Background: Actinic Keratosis (AK), is the most common precancerous skin lesion induced by the excessive Ultraviolet B (UVB) and is a significant threat to the public health. UVB exposure causes oxidative DNA damage and is considered to be a significant contributor to AK and subsequent development of skin cancer. Besides, activation of p38 MAPK also plays a significant role in the development of AK.

Objective: This study aimed at the development of a nature compound which can inhibit UVB-induced AK.

Method: MTS Cell Proliferation Assay Kit was used to detect the toxicity of astragalin. HE-staining, Immunohistochemical, Western blot and Enzyme Linked Immunosorbent Assay were applied to examine the clinicopathologic feature of AK and the change of p38 MAPK signal pathway treated with astragalin under the condition of UVB in vitro and in vivo.

Results: In our clinical findings revealed that p38 MAPK, phospho-MSK1, and γ-H2AX were significantly highly expressed in human AK tissue than the normal healthy skin tissue. Moreover, in vitro studies showed that UVB induced the phospho-MSK1 and γ-H2AX in a time- and dose-dependent manner in HaCaT cells. Further, in vitro kinase assay demonstrated that astragalin could directly bind to p38 MAPK and suppress p38 MAPK activity. Furthermore, astragalin exhibited no toxicity and suppressed the UVB-induced expression of phospho-MSK1 and γ-H2AX by suppressing p38 MAPK activity in a time-dependent and dose-dependent manner in HaCaT cells. The in vivo studies with animal UV model demonstrated that astragalin inhibited UVB-induced expression of MSK1 and γ-H2AX in Balb/c mice.

Conclusion: These results suggested that p38 MAPK is a direct valid molecular target of astragalin for the attenuation of UVB-induced AK. Furthermore, astragalin could be a potential promising novel natural therapeutic agent for the prevention and management of UVB-induced AK with high target specificity and low toxicity.

Keywords: Actinic keratosis, UVB, astragalin, p38 MAPK, phosphorylation, H2AX

1. INTRODUCTION

Actinic Keratosis (AK) is one of the most common potential precursor lesions to cutaneous Squamous Cell Carcinoma (cSCC). AK lesions predominantly occur in ultraviolet (UV)-exposed surfaces of the body, including the face, neck, scalp, hands, and lower legs [1, 2]. UV radiation is classified, depending on the wavelength, into three energy subtypes; short-wave UV (200-280 nm), mid-wave UVB (280-320 nm), and long-wave UVA (320-400 nm). All of the UVC radiation was dissipated by the ozone of the atmosphere. Approximately 95% of the UVA and 5% UVB radiation reached the Earth’s surface [3]. However, low-energy UVA radiation is weakly absorbed by canonical DNA bases and causes relatively slight direct DNA damage, while UVB is readily absorbed by DNA and causes direct lethal DNA damage [4]. Thus, UVB is considered as a major contributor to sunlight-induced AK [5]. Since the long-term UV radiation exposure is associated with the risk of AK and AK incidence is directly correlated with cumulative UV exposure; thus, AK is more common in patients aged 50 years and older [6]. However, the signaling pathways involved in UVB-induced skin diseases are the focus of the current studies and have not been completely elucidated.

The Mitogen-Activated Protein Kinases (MAPKs) belongs to the family of serine/threonine protein kinases including c-Jun N-terminal Kinases (JNKs), extracellular regulated protein kinases 1/2 (ERK1/2) and p38 MAPK, which convert extracellular stimuli, including stress into a wide range of cellular responses. JNKs and p38 MAPKs were recognized to be activated in response to environmental stresses including proinflammatory cytokines, DNA damage, oxidative stress or UV irradiation and participate in a variety of intracellular signaling cascades that regulate various cellular processes, including adaptation to stress, proliferation, differentiation, or apoptosis [7]. Recently, studies demonstrated that p38 MAPK activation is the most effective processes involved in UV-induced skin cancer [8]. Moreover, p38 MAPK was the up-stream kinase of Mitogen and Stress Activated Protein Kinase (MSK1) which also contributed to UVB-induced skin carcinogenesis [9, 10]. Furthermore, phosphorylated H2AX (γ-H2AX) has been suggested as a biomarker of DNA damage [11]. Besides, there is evidence indicating that p38 MAPK also phosphorylates H2AX directly in vitro and in vivo [12, 13]. Thus, inhibition of p38 MAPK pathway activation could prove beneficial for treating and preventing skin tumorgenesis induced by UVB irradiation.

Flavonoids, an important class of natural products with variable phenolic structures, are widely found in fruits, vegetables, and beverages, and reported to exhibit antitumor, antioxidant, and anti-
inflammatory properties coupled with their capacity to modulate critical cellular enzyme functions [14, 15]. Notably, their antitumor activity has been extensively investigated as a potential dietary-induced cancer prevention strategy against carcinogenesis. Astragalin, a natural bioactive flavonoid, isolated from persimmon, Rosa agrestis, green tea seeds and other plant sources, has been suggested to exhibit anticancer properties [16, 17]. However, the molecular mechanisms leading to cancer chemoprevention remains mostly unknown. Recently, we reported that astragalin suppressed UVB-induced activation of signal transduction pathways by blocking p38 MAPK in vitro and in vivo. Thus, astragalin acts as an inhibitor of p38 MAPK and expected to have potentially beneficial effects in the treatment and prevention of UV-induced AK.

2. MATERIALS AND METHODS

2.1. UV Model, Antibodies, and Reagents

UVB lamps were procured from Q-Lab Corporation (Cleveland, OH). Commercial astragalin (purity ≥98%) was obtained from Tong Tian Biotechnology (Shanghai, China). The antibodies against phospho-p38 MAPK (Tyr180/Tyr182), total p38 MAPK, phospho-MSK1 (Ser360), total MSK1, phospho-H2AX (Ser139), total H2AX and β-actin were purchased from Cell Signaling Technology (USA). The active p38 MAPK and inactive GST-H2AX proteins for the kinase assay were obtained from Millipore (Billerica, MA, USA). The pGEX-GST-H2AX, pLKO-shp38 plasmids were purchased from Addgene Inc. All other reagents were of standard biochemical quality and were procured from commercial suppliers.

2.2. Clinical Specimens' Collection

A total of twenty specimens of AK lesion tissue and eighteen adjacent normal skin tissues were collected from patients who had been previously diagnosed with AK at Xi'jing Hospital, Fourth Military Medical University, Shan'xi, China. Written informed consents were obtained from all the patients. The study protocols were approved by the Ethics Committee of the Xi'jing Hospital, Fourth Military Medical University, China.

2.3. Cell Culture

The human skin keratinocytes HaCaT cell lines were purchased from American Type Culture Collection (ATCC, USA) and were cultured in DMEM supplemented with 10% Fetal Bovine Serum (FBS) at 37°C in a humidified incubator under 5% CO2 atmosphere. The cell cultures were maintained by following the ATCC instructions.

2.4. MTS Assay

To assess cell proliferation and viability, the MTS-based assay was performed using CellTiter 96 AQueous One Solution Cell Proliferation Assay Kit (Promega Corporation, Madison, WI, USA). Briefly, cells were seeded (8x10^3 cells per well) into 96-well plates and incubated for 24h at 37°C with 5% CO2, according to the manufacturer's protocol. Then the cells were subsequently treated with astragalin for the indicated time. Post-incubation, 20 µl MTS solution was added to each well and incubated for 1-3h at 37°C with 5% CO2. The absorbance was measured at 490 nm to evaluate the number of viable cells using a microplate reader (Thermo Scientific). The absorbance was measured at 490 nm. At least three independent experiments were performed.

2.5. Western Blot

HaCaT cells were cultured in 6-cm dishes for 24h, and then starved in serum-free medium for 12h, then treated with different concentrations of astragalin for a different time before treatment with UVB light. The harvested cells were lysed using ice-cold RIPA lysis buffer supplemented with protease. The protein concentrations in HaCaT cells lysates were determined using the Bradford BCA method. The protein lysates were separated on 10% SDS-PAGE. For immunodetection, resolved proteins were transferred onto a Polyvinylidene Difluoride (PVDF) membrane (Millipore, USA) in a semidyblotter (Bio-Rad) for 2h using transfer buffer. The membranes were then blocked using milk-based blocking buffer (5% BSA), then the membrane was incubated with indicated primary antibodies raised in rabbit (1:1000) at 4°C overnight. The membranes were incubated with horseradish peroxidase (HRP)- conjugated secondary antibody (mouse anti-rabbit 1:3000), the protein bands were analyzed using the chemiluminiscence system (BIO-RAD, USA).

2.6. Lentiviral Infection

The lentiviral expression vectors consisted of shp38 or shMock, and packaging vectors included pMD2.G and psPAX. To prepare p38 viral particles, each viral vector and packaging vectors (pMD2.G and psPAX) were transfected into HEK293T cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The viral particles were harvested and infected into 60% confluent HaCaT cells overnight. The cell culture medium was replaced with fresh complete growth medium after 24 hours, and the cells were selected using puromycin (1.5 mg/mL) after 36 hours. The selected cells were used for experiments after confirming expression by Western blot.

2.7. In Vitro Kinase Assay

Active p38 MAPK, inactive GST-H2AX fusion proteins, ATP, and astragalin were used for the in vitro kinase assay. The reactions were performed using 1X kinase buffer containing 100 µM ATP. After incubation at 30°C for 30 minutes, the reaction was terminated, and proteins were detected by western blot.

2.8. In Vitro Pull-down Assay

HaCaT cell lysates (1 mg) were incubated with the Sepharose 4B alone, or astragalin-Sepharose 4B in the reaction buffer [50 mM Tris (pH 7.5), 5 mM Ethylenediaminetetraacetic acid (EDTA), 150 mM NaCl, 1 mM dithiothreitol (DTT), 0.01% Nonidet P-40 (NP-40), 2 µg/ml Bovine Serum Albumin (BSA), 0.02 mM phenylmethylsulfonyl Fluoride (PMSF) and 1 µg/ml protease inhibitor mixture]. After gentle rocking at 4°C overnight, the beads were washed five times with wash buffer [50 mM Tris·HCl (pH7.5), 5 mM EDTA, 150 mM NaCl, 1 mM DTT, 0.01% NP-40, and 0.02 mM PMSF] and proteins were analyzed by western blot using p38 MAPK antibody. All the experiments were performed in triplicate.

3. ANIMAL STUDY

All animal experiments were conducted according to the guideline and approved by the Animal Care Committee of Xi'jing Hospital, Fourth Military Medical University, China. Baby/c mice were acclimated for 2 weeks and shaved 24 hours before the experiment. For each experiment, mice were divided into three groups: vehicle group (n=10), UVB group (n=10), 50 mg/kg astragalin and UVB group (n=10). In the vehicle group, the dorsal skin of mice was smeared with acetone for 3h. In the UVB group, the dorsal skin of mice was smeared with acetone for 3h and then the mice were exposed to 10 KJ/m2 UVB. In the UVB group, the dorsal skin of mice was smeared with acetone for 3h and then the mice were exposed to 10 KJ/m2 UVB. The mice were sacrificed, and dorsal skin tissue samples were harvested from the same area from the back of the mice. Then the tissue specimens were immediately fixed in 4% paraformaldehyde and for Hematoxylin and Eosin (H&E) staining and Immunohistochemistry (IHC). For IHC, primary antibodies against anti p-MSK1 (Ser360) and anti γ-H2AX (Ser139) were diluted at 1:200. Anti-
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4. RESULTS

4.1. p38, Phospho-MSK1, and γ-H2AX are Overexpressed in Human AK

UVB-induced oxidative damage can lead to a number of skin-related issues including premature skin aging (photoaging) and skin cancer (photocarcinogenesis) [18]. Previous studies showed that UVB is significantly associated with UVB-induced AK [19]. The defining histopathologic feature of UVB-induced AK is umbrella-like appearance [20]. AK also showed the infiltration of inflammatory cells, and continuous disorganized growth of cuticle cells, which interrupts differentiation and leads to a thickened stratum corneum with retained nuclei [21]. A total of twenty specimens of AK lesion tissue and eighteen adjacent normal skin tissues were collected for the present study. The H&E staining evaluation revealed that the hyperkeratosis, umbrella-like appearance, infiltration of inflammatory cells, and thickness of epidermis were prominent in AK as compared to the control group (Fig. 1A). The IHC staining analysis indicated that the level of p38 (Fig. 1B), phospho-MSK1 (Fig. 1C), and γ-H2AX (Fig. 1D) in AK were elevated than that in the control group. Table 1 represented the summarized data. Next, the relationship between p38 MAPK signaling pathway and UVB-induced AK were evaluated in vitro and in vivo.

| Antigen | n  | Positive Staining (%) |
|---------|----|------------------------|
| p38     | 20 | 15 (75)                |
| normal  | 18 | 3 (17)                 |
| p-MSK1  | 20 | 17 (85)                |
| normal  | 18 | 2 (11)                 |
| γ-H2AX  | 20 | 19 (95)                |
| normal  | 18 | 1 (5)                  |

Fig. (1). The expression level of p38, phospho-MSK1, and γ-H2AX were overexpressed in human AK. (A) HE staining of the pathological changes in AK. (B-D) Immunohistochemical analysis for the detection the level of p38, phospho-MSK1 and γ-H2AX in human AK tissues (n=20) and normal healthy skin tissues (n=18). Bar: 40 μm.
were reached its highest peak at 15 min after exposure to 1 KJ/m² UVB (Fig. 2B). Therefore, the cells were treated for 15 min after exposure to 1 KJ/m² UVB in the following experiment. Further we treated the stable cell lines with knocked down p38 after exposure to 1 KJ/m² UVB in HaCaT cells. The expression of p38 was noticeably decreased after knocking down p38 as compared to the control group (Fig. 2C). Moreover, the expression level of phospho-MSK1 and γ-H2AX in shp38 HaCaT cells were significantly reduced as compared to shMock cells (Fig. 2D). The above data indicated that p38 mediated the UVB-induced MSK1 and H2AX activity. Furthermore, the cytotoxicity of UVB was evaluated by MTS assay in HaCaT cells. However, UVB (0.1, 0.2 0.5, 1, and 1.5 KJ/m²) did not reduce the viability of HaCaT cells (Fig. 2E) as compared to the absence of UVB irradiation.

4.3. Astragalin Directly Binds to p38 MAPK and Suppresses p38 MAPK Activity in vitro

To identify a potent inhibitor of p38 MAPK, we examined more than a dozens of compounds which exhibit anti-tumor effect using the in vitro kinase assay. Next, we identified astragalin as a perfect inhibitor of p38 MAPK. The chemical structural formula of as-
Astragalin was illustrated in Fig. (3A). Further, to determine the effects of astragalin on p38 MAPK activity, H2AX was used as the substrate of p38 MAPK. Astragalin strongly inhibited the phosphorylation of H2AX in a dose-dependent manner (Fig. 3B).

The in vitro kinase assay results indicated that astragalin blocked the active site of p38 MAPK, which implied the direct binding of astragalin and p38 MAPK. To verify this hypothesis, we performed an in vitro binding assay using astragalin-conjugated Sepharose 4B beads using HaCaT cells lysate. The results showed that astragalin could directly band with p38 MAPK protein (Fig. 3C). Therefore, these results revealed that astragalin directly binds to p38 MAPK and inhibits p38 MAPK activity.

Furthermore, the cytotoxicity of astragalin was evaluated by MTS assay in HaCaT cells. Astragalin (20, 50, 100, 200 µM) did not reduce the viability of HaCaT cells (Fig. 3D). Hence, astragalin does not exert any effect on HaCaT cells.

### 4.4. Astragalin Reduce UVB-induced the Downstream Signaling Pathway of p38 and DNA Damage in a Time- and Dose-dependent Manner in the HaCaT Cells

To further confirm whether astragalin could suppress UVB-induced changes of MSK1 and H2AX which are the downstream signaling molecules, HaCaT cells were treated with different concentration of astragalin (10, 20, 50 µM) at different time points (3, 6, 9h) before 1 KJ/m² UVB stimulation. SB203580, a well-known inhibitor of p38 was used as a positive control in the experiment. As demonstrated in Fig. (4), astragalin inhibited MSK1 and H2AX phosphorylation in a time- and dose-dependent manner in HaCaT cells. For instance, 50 µM astragalin induced inhibition of p38, MSK1 and H2AX phosphorylation similar to SB203580 inhibitor (Fig. 4A). These results indicated that astragalin inhibited p38 MAPK activity, and blocked UVB-induced the production of p-MSK1 and γ-H2AX. γ-H2AX is the marker of DNA damage [11]. Thus, astragalin also inhibited UVB-induced DNA damage.

### 4.5. Astragalin Prevents AK Caused by UVB Irradiation in Mouse Skin

To determine the effect of astragalin on UVB-induced AK in Balb/c mouse, adult Balb/c mice were sheared and smeared with 50 mg/kg astragalin. H&E data revealed the epidermis thickness and significantly increased infiltration of immunocytes in the 10 KJ/m² UVB group as compared to the vehicle treated group (Fig. 5A upper panel: middle column versus left column). After treatments with 50 mg/kg astragalin, the thickness of the epidermis of mice were significantly reduced, and the infiltration of immunocytes was also reduced as compared to the UVB group (Fig. 5A upper panel: right column versus middle column). Further, the p38 MAPK downstream substrates MSK1 and H2AX were detected by IHC staining. The results indicated that the expression levels of phospho-MSK1, γ-H2AX were significantly elevated after 1 KJ/m² UVB irradiation, and the expression levels were significantly reduced after treatment with astragalin respectively (Fig. 5A). Quantification of the epidermal thickness and the expression of phospho-MSK1, and γ-H2AX were analyzed by Image-Pro Plus software (Fig. 5B and 5C, P<0.01). Besides, the infiltrations of the inflammatory factor TNF-α induced by UVB in the mouse skin tissues were also detected. The results indicated that astragalin inhibited the UVB-induced production of TNF-α in vivo (Fig. 5D). Thus, these results indicated that astragalin might protect mouse skin from UVB-induced AK by suppressing p38 MAPK signaling pathway in vivo.
DISCUSSION

Excessive exposure to UV radiation induces several types of DNA damage to the skin including inflammation. It is considered a significant risk factor that leads to premature aging or photoaging and immune suppression, which further increases the risk of developing skin cancer. AK is the most common precursor lesion to skin cancer induced by UVB irradiation [22]. 5-FU, imiquimod, and solaraze are the most widely used topical agents to treat AK. Although 5-FU can inhibit RNA and DNA synthesis, its use is limited due to its high cost and side effects [25]. Therefore, it is imperative to identify novel natural compounds with higher target specificity and lower toxicity as potential therapeutic agents for the effective management of AK.

Moreover, single-target drugs have been extensively studied over the past decades for selective ligands to prevent various side effects [26]. In the present study, we demonstrated that astragalin inhibits p38 MAPK kinase by directly binding to p38 MAPK. Astragalin is a flavonoid that exhibits a wide range of pharmacological activities, including antioxidant [27], anti-inflammatory [28], and antitumor activities [29]. Nevertheless, previous studies have also recommended the use of sunscreens as essential means of protecting against UV-induced skin damage [30]. Therefore, astragalin might serve as a promising chemopreventive agent to be used in sunscreen for the prevention of UV-mediated cutaneous damage and skin carcinomas.

Moreover, the present study indicated that p38 MAPK is the direct target of astragalin by in vitro kinase assay. The p38 MAPK belongs to the MAPKs family, it plays a significant role in cellular responses induced by external stress signals, such as inflammation, proliferation, metastasis, migration, and invasion [31]. Previous studies suggested that p38 MAPK plays a crucial role in UV-induced skin carcinoma; however, inhibition of p38 MAPK has been shown to produce fewer and smaller tumors in models of solar...
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**A**

UVB (10 KJ/m²)  
Astragalin (50 mg/kg)

H&E  
p-MSK1  
γ-H2AX

**B**

| UVB (10 KJ/m²) | Astragalin (50 mg/kg) |
|---------------|----------------------|
|               | −                    |
| Astragalin    | +                    |
|               | −                    |
|               | +                    |

**C**

| UVB (10 KJ/m²) | Astragalin (50 mg/kg) |
|---------------|----------------------|
|               | −                    |
| Astragalin    | +                    |
|               | −                    |
|               | +                    |

**D**

| UVB (10 KJ/m²) | Astragalin (50 mg/kg) |
|---------------|----------------------|
|               | −                    |
| Astragalin    | +                    |
|               | −                    |
|               | +                    |

**Fig. (5).** Astragalin suppresses UVB-induced AK *in vivo*. (A) Astragalin inhibited UVB-induced epidermis thickness and the infiltration of immunocytes. Adult Balb/c mice were exposed to 10 KJ/m² UVB irradiation after administration of astragalin or acetone on the dorsal skin for 3h, and the skin was harvested at 12 h after UVB irradiation and stained with H&E and IHC. Bar: 20 µm. (B) Epidermal thickness was quantified from Fig. (5A). (C) Quantification of expression of phospho-MSK1, γ-H2AX were analyzed by the Image-Pro Plus software. (D) Astragalin inhibited the secretion of TNFα induced by 10KJ/m² UVB in mouse skin tissues. The concentrations of TNFα were determined by ELISA. The asterisks (*) indicated a significant difference as compared to control group (P<0.05). The pound (#) indicated a significant difference as compared to UVB group (P<0.05).

UV-induced skin cancer [8]. Besides, activation of p38 MAPK signaling pathways plays a critical role in the invasive phenotype of transformed squamous epithelial cells, suggesting that p38 MAPK as a target to specifically suppress cutaneous squamous epithelial cells invasion [32]. AK is the most common precancerous lesions to cSCC. Particularly, AK progress to cSCC at a rate estimated of 0.025% to 16% for an individual lesion per year [33, 34]. Our clinical data showed that p38 MAPK exhibit higher expression in AK. Therefore, inhibition of p38 MAPK represents a most promising strategy for attenuating AK.

To further elucidate the underlying mechanism of astragalin attenuating p38 MAPK activities, MSK1 and H2AX were identified *in vitro* and *in vivo*. The results of the *in vitro* study indicated that astragalin could inhibit UVB-induced phosphorylation of MSK1 and H2AX in a dose-dependent and time-dependent manner in HaCaT cells. Similar inhibition of UVB-induced expression of phospho-MSK1 and γ-H2AX by astragalin was demonstrated in the *in vivo* experiments. Besides, studies have detected inflammation in a subset of cSCC and its precursors, AK [35]. Similarly, the findings of the present study based on ELISA also suggested elevated levels of an inflammatory factor, TNF-α in UVB-induced AK; however, treatment with astragalin significantly inhibited the secretion of TNF-α in mouse skin.

**CONCLUSION**

In conclusion, these results suggested that p38 MAPK is a direct specific molecular target of astragalin for the attenuation of UVB-induced AK. Furthermore, astragalin could be a potential promising novel natural therapeutic agent for the prevention and management of UVB-induced AK with high target specificity and low toxicity.

**ETHICS APPROVAL AND CONSENT TO PARTICIPATE**

The study protocols were approved by the Ethics Committee of the Xi’jing Hospital, Fourth Military Medical University, (permit number: 2014-001), China.

All animal experiments were conducted according to the guideline and approved by the Animal Care Committee of Xi’jing Hospital, Fourth Military Medical University, China.

**HUMAN AND ANIMAL RIGHTS**

All humans research procedures followed were in accordance with the standards set forth in the Declaration of Helsinki <https://www.wma.net/policies-post/wma-declaration-of-helsinki-ethical-principles-for-medical-research-involving-human-subjects/> principles of 1975, as revised in 2008 (http://www.wma.net/en/20activities/10ethics/10helsinki/).
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