

## Inhibitory effect of Korean Red Ginseng on melanocyte proliferation and its possible implication in GM-CSF mediated signaling

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Korean Red Ginseng (KRG) has been reported to exert anticancer, anti-oxidant, and anti-inflammatory effects. However, there has been no report on the effect of KRG on skin pigmentation. In this study, we investigated the inhibitory effect of KRG on melanocyte proliferation. KRG extract (KRGE) at different concentrations had no effect on melanin synthesis in melan-A melanocytes. Saponin of KRG (SKRG) inhibited melanin content to 80% of the control at 100 ppm. Keratinocyte-derived factors induced by UV-irradiation were reported to stimulate melanogenesis, differentiation, proliferation, and dendrite formation. In this study, treatment of melan-A melanocytes with conditioned media from UV-irradiated SP-1 keratinocytes increased melanocyte proliferation. When UV-irradiated SP-1 keratinocytes were treated with KRGE or SKRG, the increase of melanocyte proliferation by the conditioned media was blocked. Granulocyte-macrophage colony-stimulating factor (GM-CSF) was produced and released from UV-irradiated keratinocytes. This factor has been reported to be involved in regulating the proliferation and differentiation of epidermal melanocytes. In this study, GM-CSF was significantly increased in SP-1 keratinocytes by UVB irradiation (30 mJ/cm<sup>2</sup>), and the proliferation of melan-A melanocytes increased significantly by GM-CSF treatment. In addition, the proliferative effect of keratinocyte-conditioned media on melan-A melanocytes was blocked by anti-GM-CSF treatment. KRGE or SKRG treatment decreased the expression of GM-CSF in SP-1 keratinocytes induced by UVB irradiation. These results demonstrate that UV irradiation induced GM-CSF expression in keratinocytes and KRGE or SKRG inhibited its expression. Therefore, KRG could be a good candidate for regulating UV-induced melanocyte proliferation.

**Keywords:** *Panax ginseng*, Korean Red Ginseng, Ultraviolet radiation, Skin pigmentation, Granulocyte-macrophage colony-stimulating factor

### INTRODUCTION

The root of ginseng (*Panax ginseng* Meyer) has been known as folk medicine in East Asian countries since time immemorial, and is now one of the most largely used herbal drugs in the world [1,2]. Korean Red Ginseng (KRG) is a herb cultivated and aged for 4 to 6 yr or more and requires extensive cleaning, steaming, and drying

processes before use [3]. In Korea, KRG is widely used, and frequently as a health supplement in food including beverages, candy, jellies, and snacks [4,5]. The active components in ginseng include ginsenosides, polysaccharides, peptides, polyacetylenic alcohols, vitamins, minor elements, and enzymes [6,7]. KRG has been shown to

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have several pharmacological functions, such as memory enhancing activities [8], antihypertensive [9], antitumor [10], antistress [11], antidiabetic [12], potentiation of erectile response [13], and aphrodisiac [14] functions. Previous studies of KRG's effects on skin were related to atopic dermatitis [15] and anti-aging [16], anti-oxidant [17], and anti-inflammatory [18] activities. However, the effects of KRG on skin pigmentation have not been investigated.

Hyperpigmentary disorders such as chloasma and freckles are due to abnormally increased epidermal melanin [18-21]. Some of the principle causes of hyperpigmentation are exposure to UV radiation, genetic factors, metabolism, inflammation, infection, the endocrine system, and scars [22,23]. Hyperpigmentation generally results from three major steps in the epidermis: proliferation of melanocytes, synthesis and activation of tyrosinase to produce melanin, and transfer of melanosomes to keratinocytes [24,25]. Numerous studies have reported on hyperpigmentation induced by UV radiation [26,27]. UV radiation is the most powerful and well-known extrinsic factor that enhances skin pigmentation [28]. Thus, UV radiation has many adverse effects on human skin, including cancer, immunosuppression, erythema, hyperpigmentation, and photo-aging [29-33].

UV irradiation increases proliferation, dendritogenesis, and melanogenesis of mouse and human melanocytes [34-38]. UV radiation that penetrates the epidermis stimulates keratinocytes to produce inflammatory cytokines and factors such as interleukin (IL)-1 [39], IL-10 [40], tumor necrosis factor (TNF)- $\alpha$  [41], basic fibroblast growth factor [42], endothelin-1 [43],  $\alpha$ -melanocyte-stimulating hormone [44], stem cell factor [45], and granulocyte macrophage colony-stimulating factor (GM-CSF) [46]. A complicated network composed of paracrine and autocrine cytokines secreted by keratinocytes and by melanocytes plays an important role in regulating melanogenesis along with their corresponding receptors [47]. In addition, several types of cross-talk among cytokine receptor signaling pathways are involved in enhanced proliferation and melanogenic activities of melanocytes.

Hyperpigmentation is clinically observed in response to inflammation [48]. Many research groups are investigating the regulation of skin pigmentation with the goal of developing hypopigmentation and/or tanning cosmetics and also to elucidate the mechanisms of pigmentary disorders to cure and/or prevent such diseases. In the present study, we examined the inhibitory effect of KRG on UV-induced pigmentation in immortalized mouse keratinocytes (SP-1 cells) and immortalized mouse melanocytes (melan-A cells).

## MATERIALS AND METHODS

### Compound and reagents

Total extract and saponin of KRG were provided by the Korea Ginseng Corporation (Daejeon, Korea). Phenylthiourea (PTU), Kojic acid, and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) and RPMI 1640 media were purchased from WelGENE (Daegu, Korea). Newborn calf serum (NBCS) was purchased from Gibco Invitrogen (Carlsbad, CA, USA). GM-CSF, anti-GM-CSF, and r-IgG were purchased from Abcam (Cambridge, UK).

### Cell culture

Murine melan-A melanocytes were kindly gifted from Prof. Dorothy C. Bennett (St. George's Hospital, London, UK) [49]. Melan-A cells were maintained in RPMI-1640 (WelGENE) medium supplemented with 10% FBS, 1% penicillin/streptomycin (P/S), and 200 nM PMA.

Murine SP-1 keratinocytes were derived from Sen-car mice and were generously provided by Dr. Stuart H. Yuspa (Laboratory of Cellular Carcinogenesis and Tumor Promotion, NCI, NIH). SP-1 keratinocytes were grown in Eagle's Minimum Essential Medium (EMEM) containing 0.05 mM Ca<sup>2+</sup>, 8% Chelex-treated heat-inactivated NBCS, and 1% P/S [50]. Under these conditions, the doubling time for melanocytes and keratinocytes was about two and three days, respectively.

### Cell viability assay

Melan-A melanocytes were seeded in 96 well plates ( $2 \times 10^4$  cells/well). After 24 h at 37°C, the medium was replaced with media containing total extract and saponin of KRG (SKRG) diluted to the appropriate concentrations. Control cells were treated with dimethyl sulfoxide (DMSO) at a final concentration of 0.1%. After 24 h, the media containing the compounds or DMSO was replaced with media containing 10% EZ-CyTox (Daeil Lab Service, Seoul, Korea). The cells were then incubated at 37°C for 30 min, and the absorbance was measured using a microplate reader (Tecan, Mannedorf, Switzerland) at a wavelength of 450 nm. All assays were performed in triplicate. The cytotoxic effect of each treatment was expressed as a percentage of cell viability relative to the untreated control cells.

### Melanin assay

Melan-A melanocytes were seeded in 24 well plates ( $1 \times 10^5$  cells/well) in RPMI 1640 medium containing

10% FBS, 1% P/S, and 200 nM PMA for 72 h. After washing with Dulbecco's phosphate buffered saline (DPBS), the cells were dissolved in 100  $\mu$ L of 1 N NaOH at 60°C for 30 min. Then, the lysates were measured at 470 nm using a microplate reader. The data were normalized to the protein content of the cell lysates. The cell lysates were subsequently processed for determination of the protein concentration using a BCA Protein Assay kit (Pierce Biotechnology, Rockford, IL, USA).

#### **Tyrosinase activity assay (extracted from melan-A melanocytes)**

Melan-A melanocytes were seeded in 100-mm culture dishes ( $1 \times 10^7$  cells/dish) and cultured for 24 h. The cells were removed from culture dishes and lysed in tyrosinase extract buffer and assayed for tyrosinase activity. KRG extract (KRGE) was diluted with an equal volume of 0.5 M potassium phosphate buffer (pH 6.8) and cell tyrosinase extracts were placed in the wells of 96 well plates. Then, the enzymatic assay was started by adding L-3, 4-dihydroxyphenylalanine (2 mg/mL) solution for 60 min. Instead of a sample, DMSO was added as a blank solution. The value of each measurement was expressed as the percent change from the control and normalized by the protein content. The cell extracts were subsequently processed for determination of the protein concentration using a BCA Protein Assay kit (Pierce Biotechnology).

#### **Cell proliferation assay**

SP-1 keratinocytes were seeded in 60-mm culture dishes ( $1.5 \times 10^6$  cells/dish). After 24 h, SP-1 keratinocytes were washed with DPBS and replenished with serum-free EMEM. After starvation for 24 h, SP-1 keratinocytes were washed with DPBS and exposed to UV radiation by a UV irradiation system (Vilber Lourmat, Marne la Vallee, France). After irradiation, the cells were replaced with 2% NBCS EMEM. After 24 h, the conditioned media was used to treat melan-A melanocytes seeded in 24 well plates ( $1 \times 10^4$  cells/well) for 24 to 72 h. After 24, 48, and 72 h, the media containing the compounds or DMSO was replaced with media containing 10% EZ-CyTox (Daeil Lab Service). The cells were then incubated at 37°C for 2 h, and the absorbance was measured using a microplate reader at a wavelength of 450 nm. All assays were performed in triplicate. The proliferation effect of each treatment was expressed as a percentage of cell viability relative to the non-irradiated control cells.

#### **Enzyme-linked immunosorbent assay**

Production of cytokines in SP-1 keratinocyte cells was

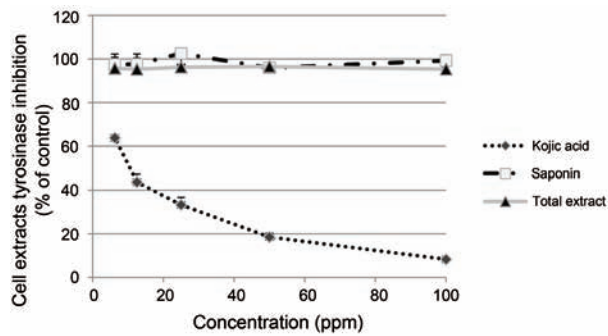
measured with an enzyme-linked immunosorbent assay (ELISA) kit (eBioscience, San Diego, CA, USA). SP-1 keratinocytes were seeded in 24 well plates ( $1.5 \times 10^5$  cells/well). After 24 h, the cells were washed with DPBS and replenished with serum-free EMEM containing total extract and SKRG. After starvation for 24 h, SP-1 keratinocytes were washed with DPBS and exposed to a radiation dose of 30 mJ/cm<sup>2</sup> of UVB (290-320 nm) light by a UV irradiation system. After irradiation, the cells were replaced with 2% NBCS EMEM containing KRGE. After 24 h, conditioned media from the UV-irradiated SP-1 keratinocytes was collected and stored at -80°C until ELISA was performed for measurement of cytokine production. Immune plates (96 well) were coated with cytokine capture antibody overnight at 4°C. The cytokine capture antibody was removed, the wells were washed 5 times with washing buffer (0.05 % Tween 20 in PBS), and then blocked with 1X assay diluent for 1 h at room temperature. The wells were washed 5 times, then 100  $\mu$ L aliquots of standard (recombinant mouse cytokine) and sample were added to each well, and the plates were incubated for 2 h at room temperature. The plates were washed 5 times with wash buffer, then cytokine detection antibody was added, and incubation was continued for 1 h at room temperature. The solution in the wells was removed and the wells were washed 5 times with wash buffer. Next, 100  $\mu$ L of avidin-HRP was added to each well, and the plates were incubated for 30 min at room temperature. The wells were washed 7 times, then 100  $\mu$ L aliquots of 1X TMB substrate solution were added to each well, and the plates were incubated for 15 min at room temperature. The color reaction was quenched with stop solution (50  $\mu$ L, 1N H<sub>2</sub>SO<sub>4</sub>). The absorbance was measured using a microplate reader at a wavelength of 450 nm.

#### **Neutralization assay**

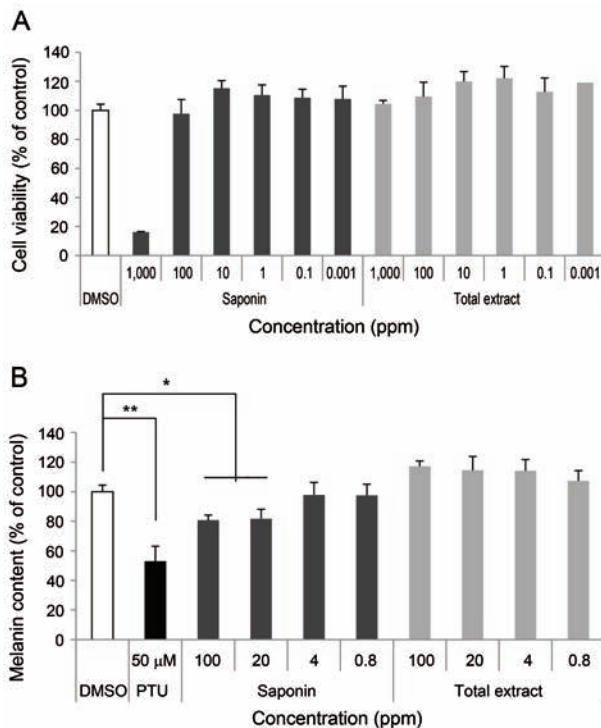
SP-1 keratinocytes were seeded in 60-mm culture dishes ( $1.5 \times 10^6$  cells/dish). After 24 h, the cells were washed with DPBS and replenished with serum-free EMEM. After starvation for 24 h, SP-1 keratinocytes were washed with DPBS and exposed to a radiation dose of 30 mJ/cm<sup>2</sup> of UVB (290 to 320 nm) light by a UV irradiation system. After irradiation, the cells were replaced with 2% NBCS EMEM containing r-IgG or anti-GM-CSF. After 24 h, the conditioned media was used to treat melan-A melanocytes seeded in 24 well plates ( $1 \times 10^4$  cells/well) for 3 d.

#### **Statistical analysis**

All values in figures are expressed as means $\pm$ SEM.

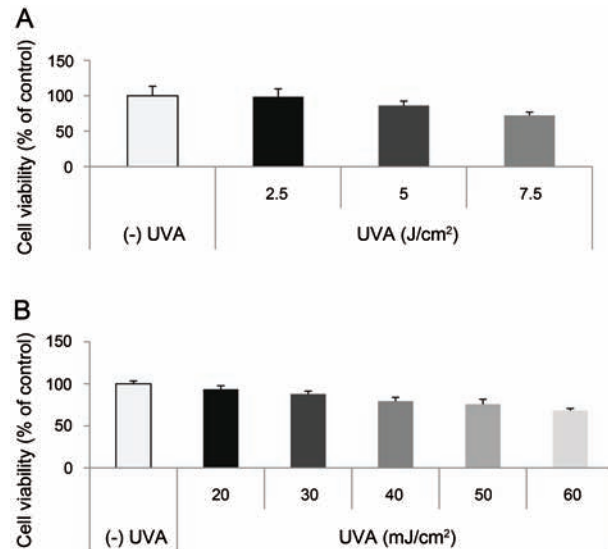


**Fig. 1.** Effect of Korean Red Ginseng (KRG) on tyrosinase activity. Total extract or saponin of KRG (100, 50, 25, 12.5, 6.25, and 3.125 ppm) was incubated with tyrosine cell extracts for 60 min at 37°C. Absorbance was measured at 490 nm. Data are shown as average±SEM. \**p*<0.05, compared with the control.



**Fig. 2.** Effect of Korean Red Ginseng (KRG) on melanin synthesis. (A) Cell viability was measured in melan-A melanocytes. Melan-A melanocytes ( $2 \times 10^4$  cells/well) were cultured in 96 well plates. After incubation with total extract or saponin of KRG (SKRG) for 24 h, cell viability was measured using a water soluble tetrazolium salt-1 assay. (B) Melan-A melanocytes ( $1 \times 10^5$  cells/well) were cultured in 24 well plates. The melanin content of the cells was measured after incubation with total extract, SKRG (100, 20, 4, and 0.8 ppm), or 50  $\mu$ M phenylthiourea (PTU) for 72 h. Data are shown as average±SEM. \**p*<0.05, \*\**p*<0.01, compared with the control. DMSO, dimethyl sulfoxide.

The analysis was conducted using one-way ANOVA's (IBM SPSS ver. 20.0; IBM, Armonk, NY, USA) and *p*-values less than 0.05 were considered statistically significant.



**Fig. 3.** Effect of UV on SP-1 keratinocyte viability. SP-1 keratinocytes were exposed to UVA at doses ranging from 2.5 to 7.5 J/cm<sup>2</sup> or UVB at doses ranging from 20 to 60 mJ/cm<sup>2</sup>. Cell viability was examined at 24 h. SP-1 keratinocyte viability following (A) UVA or (B) UVB irradiation. Data are shown as average±SEM.

## RESULTS

### Effect of Korean Red Ginseng on tyrosinase activity

To demonstrate whether KRG inhibits tyrosinase activity directly, tyrosinase activity was measured in cell lysates from melan-A melanocytes. Total lysates and SKRG did not inhibit tyrosinase activity directly in the cell lysates (Fig. 1). Kojic acid, a well known tyrosinase inhibitor was used as a positive control [51].

### Effect of Korean Red Ginseng on melanin synthesis

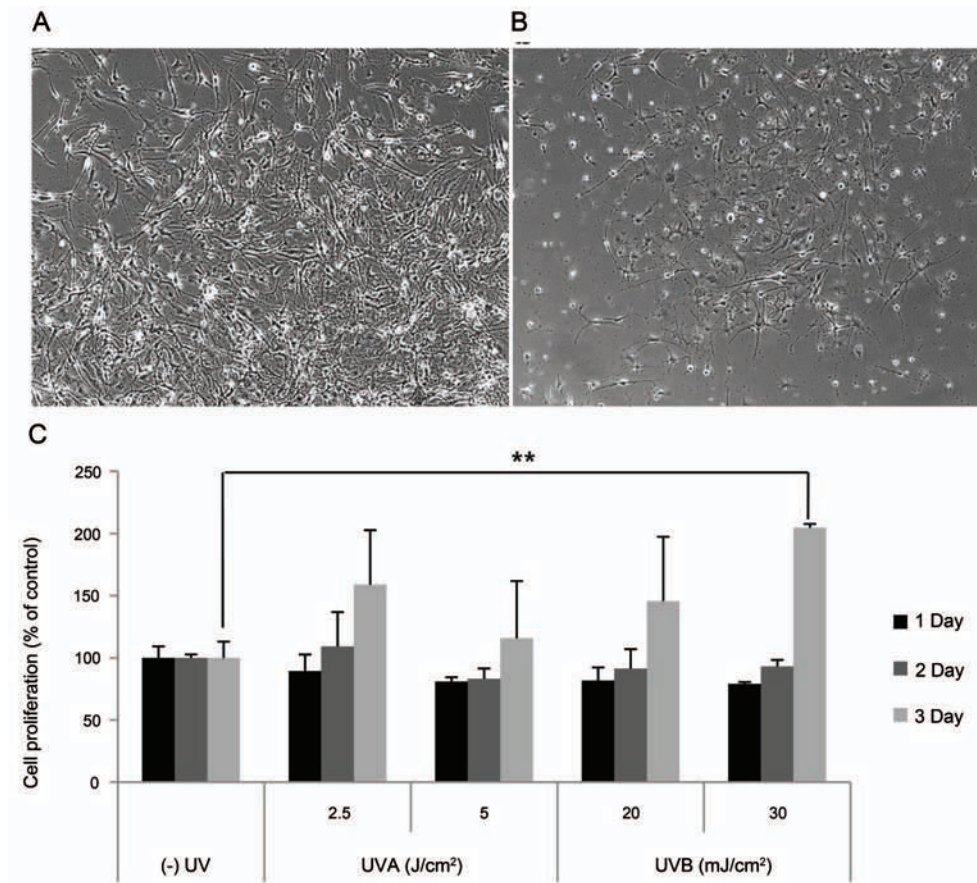
The cytotoxicity of KRGE on melan-A melanocytes was measured by the water soluble tetrazolium salt-1 assay. The cells were seeded in 96 well plates at  $2 \times 10^4$  cells/well and treated with different concentrations of each KRGE (0.001 to 1,000 ppm) for 24 h. SKRG reduced cell viability under 20% at 1,000 ppm (Fig. 2A).

To further determine whether KRGE regulates melanin synthesis, melanin content was measured after treatment with red ginseng extracts. KRGE did not inhibit melanin content at 0.8 to 100 ppm. SKRG inhibited melanin content to 80% of the control at 100 ppm (Fig. 2B).

### Effect of UV on SP-1 keratinocyte viability

To determine the dosage of UV radiation, we measured cell viability in UV-irradiated SP-1 keratinocytes. UVA irradiation with 7.5 J/cm<sup>2</sup> reduced cell viability by





**Fig. 4.** Effect of conditioned media from UV-irradiated SP-1 keratinocytes on melan-A melanocyte proliferation. SP-1 keratinocytes were exposed to UVA (2.5, 5 J/cm<sup>2</sup>) or UVB (20, 30 mJ/cm<sup>2</sup>). After 24 h, the conditioned media was used to treat melan-A melanocytes. After 72 h, melan-A melanocyte proliferation was measured as described in the Materials and Methods. (A) UVB (30 mJ/cm<sup>2</sup>) irradiated conditioned media, (B) control, and (C) cell proliferation assay. Cells were observed under a phase-contrast microscope (Olympus CKX41; Olympus Optical, Tokyo, Japan) and images were photographed using a DMCE camera (INS Industry, Seoul, Korea) and the included DMC advanced software ( $\times 100$ ). Data are shown as average  $\pm$  SEM. \*\* $p < 0.01$ , compared with the control.

up to 70% (Fig. 3A). UVB irradiation with 40 mJ/cm<sup>2</sup> reduced cell viability by up to 80% (Fig. 3B). From these results, we determined the dose of UVA from 2.5 to 5 J/cm<sup>2</sup> and UVB from 20 to 30 mJ/cm<sup>2</sup>.

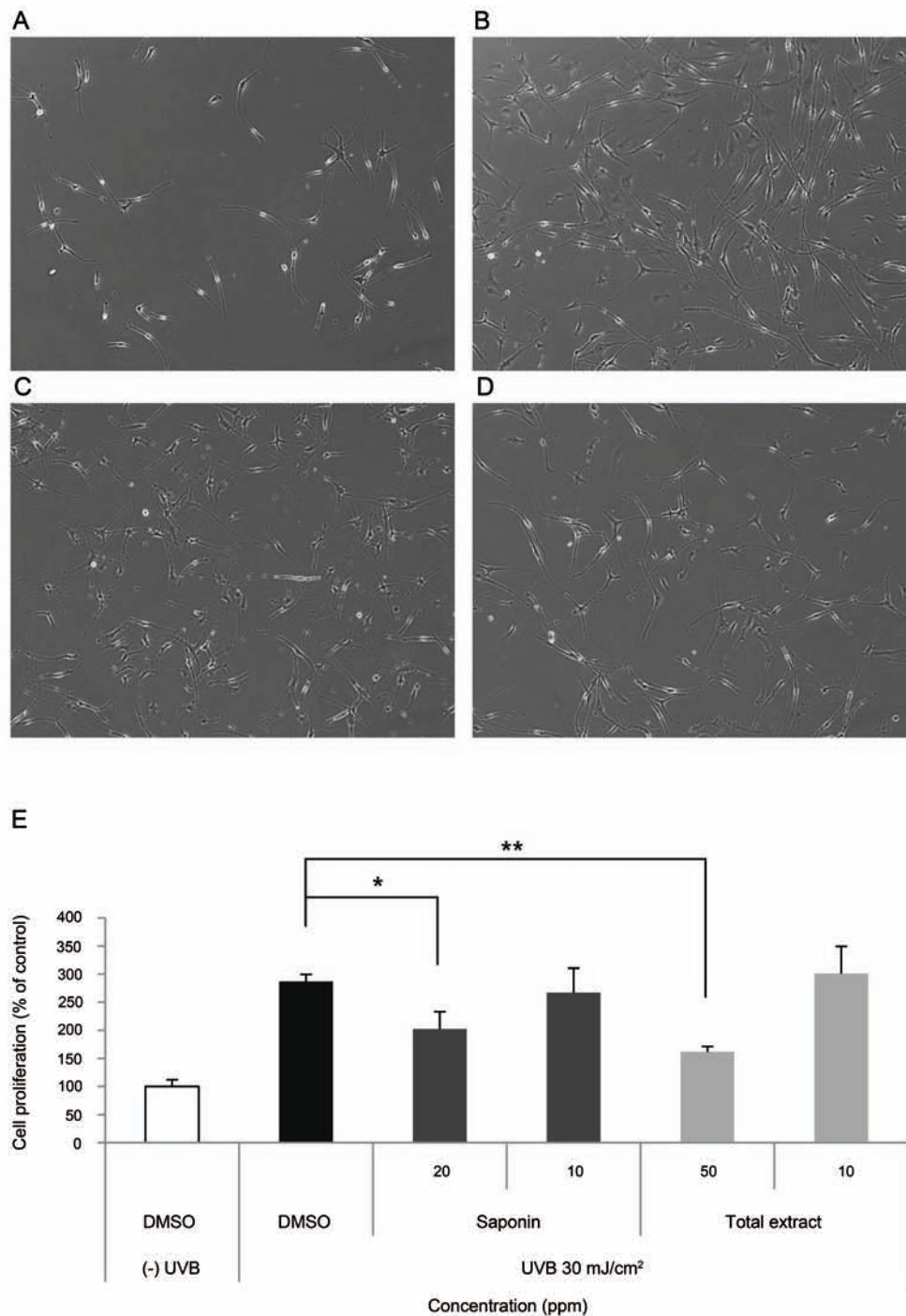
#### Effect of conditioned media from UV-irradiated SP-1 keratinocytes on melan-A melanocyte proliferation

To elucidate the effect of conditioned media from UV-irradiated SP-1 keratinocytes, we irradiated SP-1 keratinocytes with UV radiation. Then, the conditioned media from UVA (2.5, 5 J/cm<sup>2</sup>)- or UVB (20, 30 mJ/cm<sup>2</sup>)-irradiated SP-1 keratinocytes was used to treat melan-A melanocytes. Treatment with conditioned media from UV-irradiated SP-1 keratinocytes increased melan-A melanocyte proliferation. Cell density alterations of melan-A melanocytes exposed to SP-1 keratinocytes conditioned media irradiated with 30 mJ/cm<sup>2</sup> of UVB were viewed

by phase-contrast microscopy. The cell density was markedly increased in UVB irradiated SP-1 keratinocyte conditioned media treated melan-A cells (Fig. 4A), compared with the non-irradiated control (Fig. 4B) at 72h. Specifically, conditioned media from SP-1 keratinocytes irradiated with 30 mJ/cm<sup>2</sup> of UVB increased melan-A melanocyte proliferation about two-fold compared with the non-irradiated control (Fig. 4C).

#### Effect of Korean Red Ginseng on proliferation of melan-A melanocytes induced by conditioned media from UV-irradiated SP-1 keratinocytes

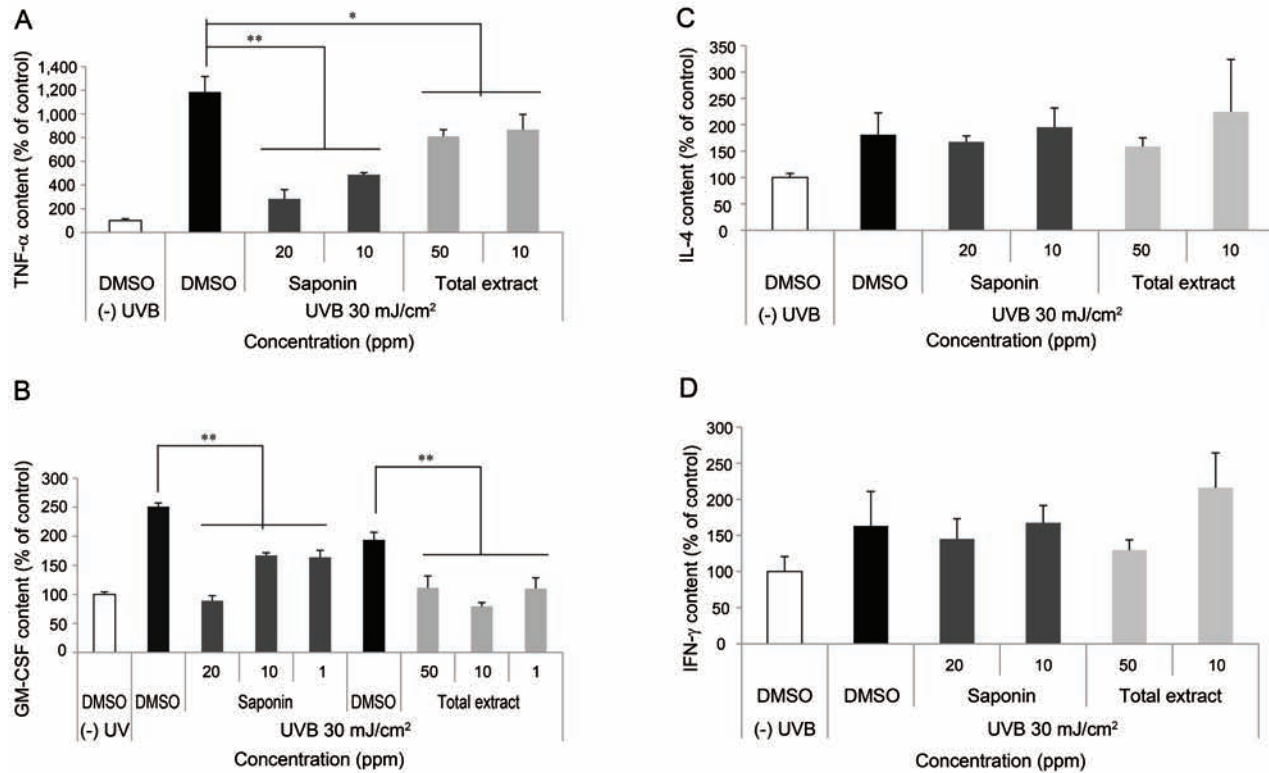
To elucidate the effect of KRG on proliferation of melan-A melanocytes induced by conditioned media from UVB-irradiated SP-1 keratinocytes, conditioned media from UVB-irradiated SP-1 keratinocytes treated with KRG was tested on melan-A melanocytes. SP-1 keratinocytes were seeded in 60-mm culture dishes ( $1.5 \times 10^6$



**Fig. 5.** Effect of Korean Red Ginseng (KRG) on melan-A melanocyte proliferation induced by conditioned media from UV-irradiated SP-1 keratinocytes. Before UV irradiation, SP-1 keratinocyte cells were treated with serum free Eagle's Minimum Essential Medium (EMEM) containing total extract or saponin of KRG (SKRG) (10, 20, and 50 ppm). After 24 h, SP-1 keratinocytes were irradiated with UVB at a dose of 30 mJ/cm<sup>2</sup>. Immediately, the cells were treated with 2% newborn calf serum EMEM containing total extract or SKRG (10, 20, and 50 ppm). (A) Control, (B) UVB (30 mJ/cm<sup>2</sup>), (C) SKRG (20 ppm), (D) total extract (50 ppm), and (E) cell proliferation assay. Cells were observed under a phase-contrast microscope (Olympus CKX41; Olympus Optical, Tokyo, Japan) and images were photographed using a DMCE camera (INS Industry, Seoul, Korea) and the included DMC advanced software ( $\times 100$ ). Data are shown as average $\pm$ SEM. DMSO, dimethyl sulfoxide. \* $p < 0.05$ , \*\* $p < 0.01$ , compared with the control.

cells/dish). After 24 h, the cells were washed with DPBS and replenished with serum-free EMEM containing total

extract and saponin of KRG. After starvation for 24 h, SP-1 keratinocytes were washed with DPBS and exposed



**Fig. 6.** Effect of Korean Red Ginseng (KRG) on the expression of cytokines in UV-irradiated SP-1 keratinocytes. Before UV irradiation, SP-1 keratinocyte were treated with serum free Eagle's Minimum Essential Medium (EMEM) containing total extract or saponin of KRG (SKRG) (10, 20, and 50 ppm). After 24 h, SP-1 keratinocytes were irradiated with UVB at a dose of 30 mJ/cm<sup>2</sup>. Immediately, the cells were treated with 2% newborn calf serum EMEM containing total extract or SKRG (10, 20, and 50 ppm). After 24 h, the conditioned media was measured by enzyme-linked immunosorbent assay as described in the Materials and Methods section. (A) Tumor necrosis factor (TNF)- $\alpha$ , (B) granulocyte macrophage colony-stimulating factor (GM-CSF), (C) interleukin (IL)-4, and (D) interferon (IFN)- $\gamma$ . Data are shown as average $\pm$ SEM, DMSO, dimethyl sulfoxide. \* $p$ <0.05, \*\* $p$ <0.01, compared with the control.

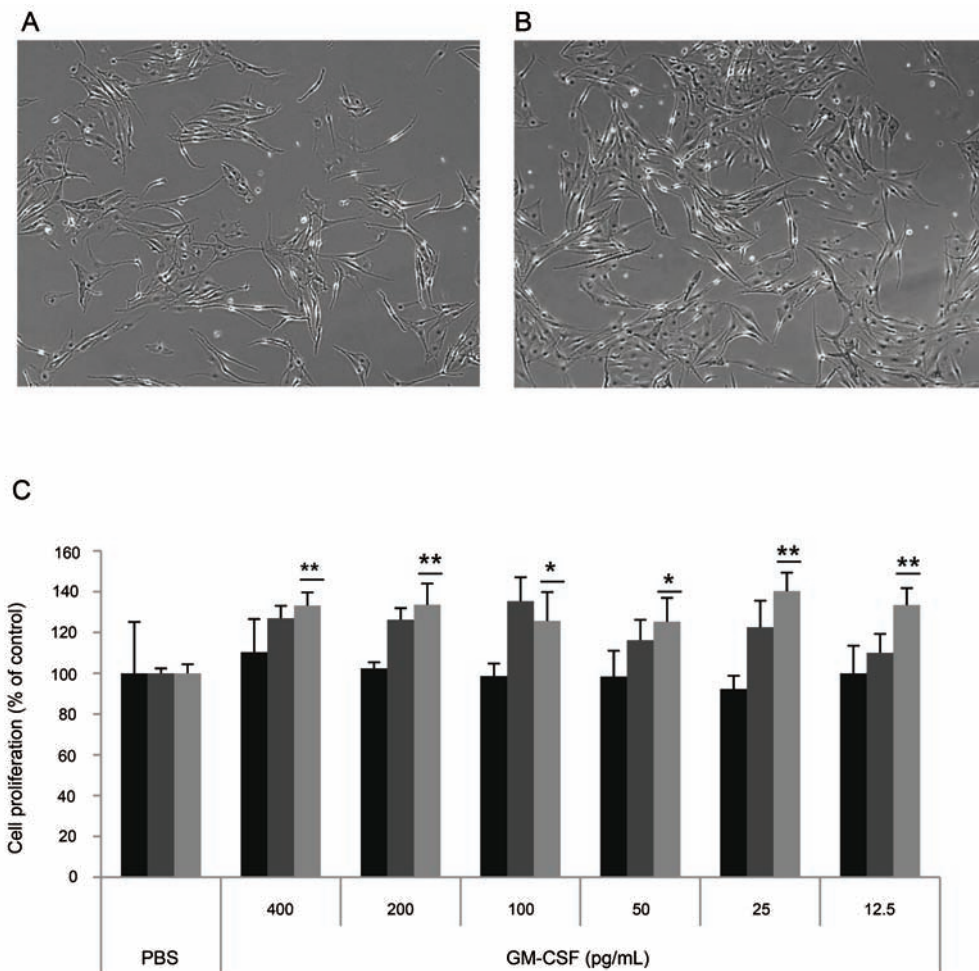
to UV radiation by a UV irradiation system. After irradiation, the cells were replaced with 2% NBCS EMEM containing total extract and saponin of KRG. After 24 h, the conditioned media was used to treat melan-A melanocytes for 24 to 72 h.

The cell density was markedly increased in UVB irradiated SP-1 keratinocyte conditioned media treated melan-A cells (Fig. 5B), compared with the non-irradiated control (Fig. 5A) at 72 h. SP-1 conditioned media treated with SKRG (Fig. 5C) or KRGE (Fig. 5D) reduced melan-A melanocyte densities compared with the UVB irradiated SP-1 keratinocyte conditioned media treated melan-A cells. Treatment of melan-A melanocytes with conditioned media from UVB-irradiated SP-1 keratinocytes increased cell proliferation by three-fold. SP-1 conditioned media treated with KRGE or SKRG inhibited melan-A melanocyte proliferation by about 50% at 50 ppm KRGE and 40% at 20 ppm SKRG, respectively (Fig. 5E).

### Effect of Korean Red Ginseng on the expression of cytokines in UV-irradiated SP-1 keratinocytes

UVB exposure stimulates the production of pro-inflammatory cytokines such as interferon (IFN)- $\gamma$ , IL-6, and TNF- $\alpha$ , which are implicated in the progression of pigmentation [52]. To elucidate the effect of KRG on UVB-induced cytokine expression in SP-1 keratinocytes, we measured the production of cytokines (IFN- $\gamma$ , IL-4, TNF- $\alpha$ , and GM-CSF) using an ELISA kit (eBioscience). UVB irradiation increased cytokine expression (IFN- $\gamma$ , IL-4, TNF- $\alpha$ , and GM-CSF) in SP-1 keratinocytes compared with the non-irradiated control. SKRG decreased expression of TNF- $\alpha$  by 75% compared with the untreated irradiated control (Fig. 6A). In addition, saponin and total KRGE decreased expression of GM-CSF by 60% compared with the untreated irradiated control (Fig. 6B). However, the expression of IL-4 (Fig. 6C) and IFN- $\gamma$  (Fig. 6D) was not changed.





**Fig. 7.** Effect of granulocyte macrophage colony-stimulating factor (GM-CSF) on melan-A melanocyte proliferation. (A) Melan-A melanocytes ( $1 \times 10^4$  cells/well) were cultured in 24 well plates. After incubation with 400, 200, 100, 50, 25, or 12.5 pg/mL GM-CSF for 24, 48, and 72 h, cell proliferation was measured as described in the Materials and Methods section. (A) Control, (B) GM-CSF (25 pg/mL), and (C) cell proliferation assay. Cells were observed under a phase-contrast microscope (Olympus CKX41, Olympus Optical, Tokyo, Japan) and images were photographed using a DMCE camera (INS Industry, Seoul, Korea) and the included DMCE advanced software ( $\times 100$ ). Data are shown as average  $\pm$  SEM. PBS, phosphate buffered saline. \* $p < 0.05$ , \*\* $p < 0.01$ , compared with the control.

#### Effect of granulocyte macrophage colony-stimulating factor on melan-A melanocyte proliferation

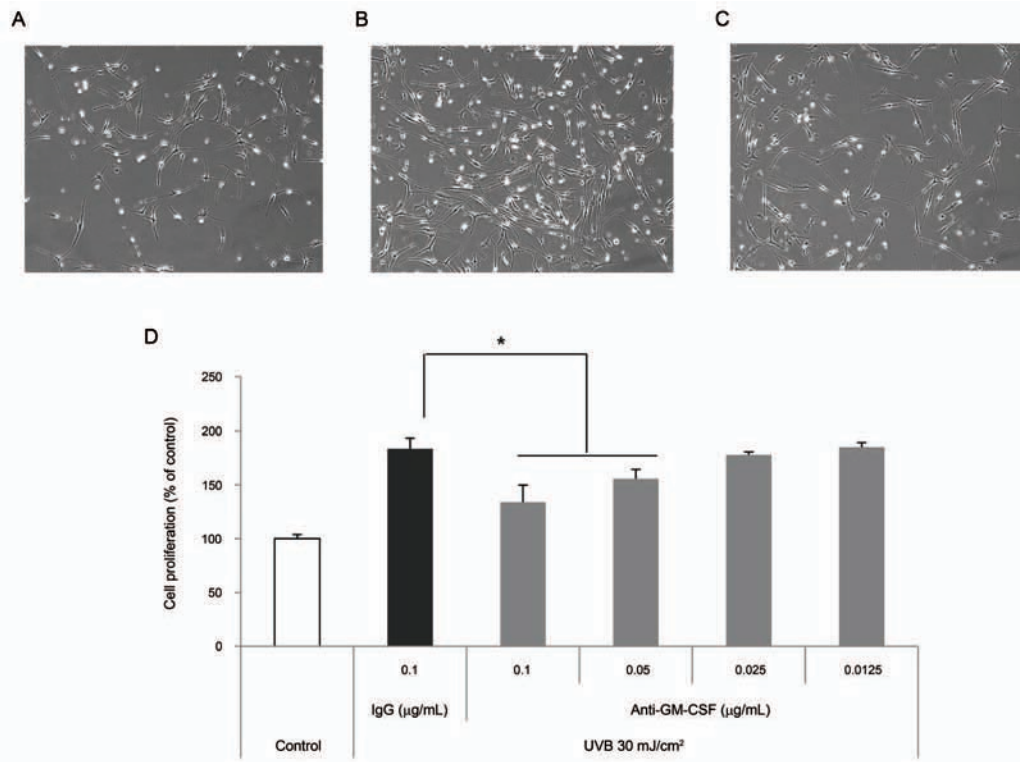
To determine whether GM-CSF increases melan-A melanocyte proliferation, cell proliferation was measured after treatment with GM-CSF (12.5-400 pg/mL) to melan-A melanocyte directly. The cell density was increased in 25 pg/mL GM-CSF treated melan-A cells (Fig. 7B) compared with control cells (Fig. 7A) at 72 h, GM-CSF treatment increased melan-A melanocyte proliferation by about 40% at 25 pg/mL (Fig. 7C).

#### Effect of granulocyte macrophage colony-stimulating factor from UVB-irradiated SP-1 keratinocytes on melanocyte proliferation

We determined whether GM-CSF from UVB-irradiat-

ed SP-1 keratinocytes involved in melan-A melanocyte proliferation by pre-treatment with neutralizing anti-GM-CSF antibodies. Conditioned media from UVB-irradiated SP-1 keratinocytes increased melan-A melanocyte proliferation by about two-fold compared with the non-irradiated control (Fig. 8A, B). When antibodies (0.1  $\mu\text{g}/\mu\text{L}$ ) against GM-CSF were added, UVB-irradiated SP-1 keratinocytes conditioned media inhibited melan-A melanocyte proliferation (Fig. 8C) compared with UVB-irradiated control (Fig. 8B). Anti-GM-CSF treatment decreased melan-A melanocyte proliferation by about 50% at 0.1  $\mu\text{g}/\mu\text{L}$  (Fig. 8D). GM-CSF-specific antibodies partially abolished the proliferating effects of UVB irradiated SP-1 keratinocyte conditioned media on melan-A melanocytes.





**Fig. 8.** Effect of conditioned media from UVB-irradiated SP-1 keratinocytes treated with anti-granulocyte macrophage colony-stimulating factor (GM-CSF) on melanocyte proliferation. SP-1 keratinocytes were irradiated with UVB at a dose of 30 mJ/cm<sup>2</sup>. Immediately, the cells were treated with r-IgG or anti-GM-CSF. After 24 h, the conditioned media was used to treat melan-A melanocytes seeded in 24 well plates (1×10<sup>4</sup> cells/well) for 3 d. (A) Control, (B) r-IgG (0.1 µg/µL), (C) anti-GM-CSF (0.1 µg/µL), and (D) cell proliferation assay. Cells were observed under a phase-contrast microscope (Olympus CKX41, Olympus Optical, Tokyo, Japan) and images were photographed using a DMCE camera (INS Industry, Seoul, Korea) and the included DMC advanced software (×100). Data are shown as average±SEM. \**p*<0.05, compared with the control.

## DISCUSSION

KRG has a wide range of pharmacological and physiological actions, which include memory enhancing activities, antihypertensive, antitumor, antistress, antidiabetic, potentiation of erectile response, aphrodisiac, atopic dermatitis, anti-aging, anti-oxidant, and anti-inflammatory activities [8-18].

Although KRG has been used as an effective agent with protective and curable roles in a variety of diseases, it has not been studied for a protective role against UV-induced pigmentation. We examined the effects of KRG on the regulation of melanocyte proliferation and key molecules involved in UV-induced pigmentation.

Hyperpigmentation is characterized by an abnormal increase of melanin in the skin [22,23]. Recently, many efforts have focused on understanding the mechanical insights of melanogenesis to develop new therapeutic agents against skin pigmentation abnormalities. Tyrosinase is a key enzyme in melanogenesis that plays a regulatory role in the production of melanin [47]. Total

extract and SKRG did not inhibit tyrosinase activity (Fig. 1). SKRG slightly inhibited melanin synthesis in melan-A melanocytes. However, KRGE did not inhibit melanin synthesis (Fig. 2). These results suggest that KRG weakly inhibits tyrosinase activity and melanin synthesis.

We showed that conditioned media from UV-irradiated SP-1 keratinocytes increased melan-A melanocyte proliferation. Specifically, conditioned media from UVB-irradiated SP-1 keratinocytes increased melan-A melanocyte proliferation about two-fold compared with conditioned media from non-irradiated SP-1 keratinocytes (Fig. 4). On the other hand, conditioned media from UVB-irradiated SP-1 keratinocytes treated with KRGE decreased melan-A melanocyte proliferation by 50% (Fig. 5).

Paracrine regulation of melanogenesis by keratinocyte-derived factors has been reported [39-46]. Some cytokines such as GM-CSF, IL-4, IFN-γ, and TNF-α are known to be elevated in keratinocytes exposed to UVB and are associated with apoptotic cell death, inflammation, and immunosuppression [53]. We found that UVB irradiation (30 mJ/cm<sup>2</sup>) significantly elevated GM-CSF,

IL-4, IFN- $\gamma$ , and TNF- $\alpha$  in SP-1 keratinocytes. The expression of GM-CSF and TNF- $\alpha$  was suppressed by KRG in a dose-dependent manner (Fig. 6).

GM-CSF [54] induces myeloid progenitor cells from bone marrow to form colonies containing granulocytes and macrophages in semisolid media. GM-CSF also acts on mature macrophages, eosinophils, and neutrophils to stimulate various functional activities [55]. GM-CSF is an acidic glycoprotein (human=18-22 kDa [55]; mouse=23 kDa [56]), which binds to high affinity receptors on GM-CSF-sensitive cells. In addition to hematopoietic cells, GM-CSF stimulates migration and proliferation of human endothelial cells [57]. Mammalian keratinocytes are capable of producing GM-CSF following external stimulation, and GM-CSF is involved in regulating the proliferation of keratinocytes in an autocrine manner [58]. GM-CSF is also involved in regulating the proliferation and differentiation of epidermal melanocytes [46]. In this study, we showed that conditioned medium from UV-irradiated SP-1 keratinocytes increased melanocyte proliferation. However, conditioned media from UV-irradiated SP-1 keratinocytes treated with KRGE decreased melan-A melanocyte proliferation. GM-CSF was produced and released from UV-irradiated SP-1 keratinocytes. The proliferation of melan-A melanocytes increased significantly by GM-CSF treatment (Fig. 7). In addition, the effect of SP-1 keratinocyte conditioned media on melan-A melanocyte proliferation was nulled by anti-GM-CSF treatment (Fig. 8). The expression of GM-CSF in UV-irradiated SP-1 keratinocytes decreased due to KRGE treatment. Thus, these results suggest that KRG inhibited melan-A melanocyte proliferation by decreasing expression of GM-CSF from UV-irradiated SP-1 keratinocytes and the inhibitory effect of KRG on GM-CSF is mainly from saponin in KRGE.

In conclusion, our results provide insights into the use of KRG for hyperpigmentary problems and skin whitening. Further studies are required to elucidate the inhibitory mechanism of KRG on GM-CSF expression in UV-irradiated keratinocytes.

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