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Effects of ginseng saponins isolated from red ginseng on ultraviolet B-induced skin aging in hairless mice

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ABSTRACT

It is well-known that chronic ultraviolet B (UVB) exposure at low-dose causes skin photoaging including increases in skin thickness and wrinkle formation and reduction in skin elasticity. This study examined the effects of total saponins and ginsenoside Rb_1 isolated from Red Ginseng roots on skin thickness, elasticity, and wrinkle formation caused by long-term, low-dose UVB irradiation in hairless mice. The topical application of total ginseng saponins (10 pg or 100 ng/mouse) and ginsenoside Rb_1 (100 fg, 10 pg, or 1 ng/mouse) significantly inhibited increases in skin thickness and wrinkle formation and the reduction in skin elasticity induced by long-term UVB irradiation. Furthermore, we examined the histological effects of total saponins and ginsenoside Rb_1 in the skin of UVB-irradiated hairless mice. The increases in apoptotic, Ki-67-, and 8-hydroxy-2′-deoxyguanosine-positive cells induced by UVB exposure were prevented by the topical application of total saponins and ginsenoside Rb_1. Furthermore, total saponins and ginsenoside Rb_1 prevented the disruption of collagen fibers induced by the long-term UVB irradiation. Ginsenoside Rb_1 (100 fg, 10 pg, and 1 ng/ml) increased the Bcl-2 expression level in UVB-treated human keratinocytes. The protective effect of ginsenoside Rb_1 on UVB-mediated apoptosis may be due to the up-regulation of Bcl-2 expression. These results suggest that the protective effect of ginsenoside Rb_1 on skin photoaging induced by chronic UVB exposure may be due to the increase in collagen synthesis and/or the inhibition of matrix metalloproteinase expression in dermal fibroblasts.

1. Introduction

Red Ginseng root (*Panax ginseng* C.A. Meyer) is used clinically in China, Korea, and Japan for various diseases including atherosclerosis, liver dysfunction, cerebrovascular diseases, hypertension, and post-menopausal disorder (Yamamoto, 1988). Recently, we reported that the promotion of burn wound healing by ginsenoside Rb_1 might be due to the promotion of angiogenesis during skin wound repair through stimulation of vascular endothelial growth factor (VEGF) production and an increase in hypoxia inducible factor (HIF)-1α expression in keratinocytes and the elevation of IL-1β from macrophage accumulation in the burn wound area (Kimura et al., 2006). Furthermore, the previous report showed that the facilitating actions of ginsenoside Rb_1 might be due to the promotion of angiogenesis via the activation of basic fibroblast growth factor (bFGF) through the increase in histamine released from mast cells recruited by the stimulation of monocyte chemoattractant protein-1 (MCP-1) as another mechanism (Kawahira et al., 2008). It is well known that an increase in skin thickness and a reduction in skin elasticity are caused by sun exposure. This phenomenon is known as photoaging and is characterized by histological changes including damage to collagen fibers and excessive deposition of abnormal elastic fibers (Sams and Smith, 1961; Smith et al., 1962; Uitto et al., 1989). We found recently that the protection by Red Ginseng extract against acute UVB-irradiated skin aging, such as the increase in skin thickness and pigmentation and the reduction in skin elasticity, might be due to the inhibition of increases in skin TGF-β1 content induced by UVB irradiation (Kim et al., 2008). This study examined the effects of total saponins and ginsenoside Rb_1 isolated from Red Ginseng roots on skin thickness, elasticity, and wrinkles caused by the long-term, low-dose UVB irradiation in hairless mice.

2. Materials and methods

2.1. Materials

Total ginseng saponins were isolated by the methods described by Shibata and co-workers (Nagai et al., 1971; Sanada et al., 1974a, b; Shibata et al., 1985; Shibata, 2001). Briefly, ginsenoside Rb_1 (Fig. 1) was isolated and purified from the total saponin fractions of roots of

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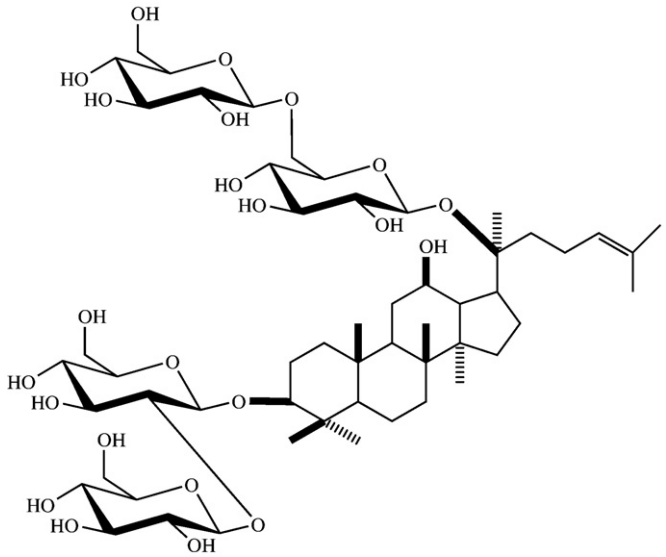

Fig. 1. The structure of ginsenoside Rb_1.

Panax ginseng C.A. Meyer, Korean Red Ginseng, by repeated column chromatography on silica gel with $CHCl_3$–MeOH–H_2O (65:35:10, v/v) and octadecylsilyl silica with MeOH–H_2O (1:1 to 7:3, v/v). The purity of ginsenoside Rb_1 used in this study was more than 99.99%, as determined by high-performance liquid chromatography. The total ginseng saponin was dissolved in ethanol–propylene glycol (7:3, v/v), at the concentrations of 200 pg and 2 μg/ml, and ginsenoside Rb_1 was also dissolved in the same solution, at the concentrations of 2 pg, 200 pg and 20 ng/ml. Sodium ascorbate (3 g) was dissolved in an ethanol–propylene glycol mixture (100 ml) and used as a positive control. Sample solutions (50 μl/mouse) were topically applied in the dorsal region. Therefore, total ginseng saponin was applied at the dose of 10 pg and 100 ng/mouse, ginsenoside Rb_1 was applied at the dose of 100 fg, 10 pg and 1 ng/mouse, and vitamin C was applied at 1.5 mg/mouse. Rat monoclonal anti-mouse Ki-67 antibody, rabbit polyclonal anti-rat biotin-labeled immunoglobulin antibody, and peroxidase-labeled streptoavidin were purchased from DakoCytomation (Kyoto, Japan). Anti-8-hydroxy-2′-deoxyguanosine (8-OHdG) (clone N45.1) and rabbit anti-mouse collagen type I were purchased from Japan Institute for the Control of Aging (Shizuoka, Japan) and CEDARLANE Lab. Lt. (Ontario, Canada), respectively. Rabbit monoclonal anti-Bcl-2 (clone 50E3), rabbit polyclonal anti-Bax, and rabbit polyclonal anti-Bak antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). Mouse monoclonal anti-β-actin antibody was purchased from Sigma. Other chemicals were of reagent grade.

2.2. Cells

Human keratinocytes, human keratinocyte basal medium (KB-2, 0.15 mM Ca^{2+}), and KG-2 medium (KB-2 medium containing 10 μg/ml of insulin, 0.1 ng/ml of human recombinant epidermal growth factor (hEGF), 0.5 μg/ml of hydrocortisone, 50 μg/ml of gentamycin, 50 ng/ml of amphotericin B, and 0.4% (v/v) bovine hypophysis extract) were purchased from Kurabo Co. (Tokyo, Japan). To evaluate the effects of natural compounds on UVB-induced biological changes, human keratinocytes are used in a medium containing a low calcium concentration (0.15 to 0.3 mM) and 80% subconfluent (Adhami et al., 2003; Onoue et al., 2003; Wang and Kochevar 2005; Ishida and Sakaguchi 2007). Therefore, we also examined the effects of ginsenoside Rb_1 on UVB-irradiated Bcl-2 and Bax expression in human keratinocytes cultured under the conditions of a low calcium concentration at 0.15 mM and 80% subconfluent.

2.3. Animals

Male albino hairless HOS: HR-1 mice (5 weeks old) were purchased from Hoshino Laboratory Animals Co. Ltd. (Saitama, Japan), housed for 1 week in a temperature-controlled room at 25 ± 1 °C and 60% relative humidity, and given free access to standard laboratory diet and water before the experiments. Mice were treated according to the Ethical Guidelines of the Animal Center, Graduate School of Medicine, Ehime University, and the experimental protocol was approved by the Animal Studies Committee of Ehime University.

2.4. Measurement of the skin thickness, elasticity, and wrinkles induced by UVB irradiation

To examine the effects of total ginseng saponins and ginsenoside Rb_1 on skin thickness, elasticity, and wrinkles induced by UVB irradiation, a UVB lamp (15 W type, UV maximum wavelength 312 nm; UV intensity 100 μW/cm^2; Ieda Boeki Co., Tokyo, Japan) was used in this study. The time of UV irradiation was varied to control the UVB energy applied to the dorsal region of each mouse. The value of the minimal erythema dose (MED) per mouse was about 36 mJ/cm^2. Total ginseng saponins (10 pg and 100 ng/mouse), ginsenoside Rb_1 (100 fg, 10 pg, and 1 ng/mouse) and 3% vitamin C (1.5 mg/mouse) were applied topically to the dorsal region of each mouse every day for 12 weeks. The initial dose of UVB was set at 36 mJ/cm^2, which was subsequently increased to 54 mJ/cm^2 at weeks 1–4, 72 mJ/cm^2 at weeks 4–7, 108 mJ/cm^2 at weeks 7–10, and finally to 122 mJ/cm^2 at weeks 10–12. The frequency of UVB irradiation was set at three times per week before the topical application of vehicle (control), the indicated amounts of total ginseng saponins, or ginsenoside Rb_1. In this protocol, wrinkles began to be observed macroscopically in the dorsal region from about 6 weeks after the initiation of UVB irradiation. The skin thickness and elasticity after UVB irradiation were measured every week using a Quick Mini Caliper (Mitutoyo Co., Kanagawa, Japan) and a Digimatic Caliper (Mitutoyo Co., Kanagawa, Japan), respectively. To evaluate the formation of wrinkles after the UVB irradiation, each hairless mouse was anesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg body weight) at 6 and 9 weeks, and then the UVB-irradiated dorsal area (site of wrinkle formation) was photographed. The degree of wrinkle formation was assessed from the photograph of each animal according to the grading scale described in Table 1, whereas the name of the animal group was kept blind; this is a modification of the method described by Bissett et al. (1987).

2.5. Measurement of TGF-β1 and glutathione in UVB-irradiated skin

At week 12, all mice were sacrificed by the overdose of pentobarbital, and then all skin tissues were quickly removed. The removed skin tissue (100 mg) was washed in phosphate buffered saline (PBS, pH 7.0) and cut into small pieces, and then tissue protein extraction reagent (T-PER) containing protease inhibitor (Pierce Co., Rockford, IL, USA) (2 ml) was added to the skin tissue and the mixture was homogenized. After the skin homogenate was centrifuged at 2000 ×g

Table 1
Grading of mouse skin wrinkles

Grade	Evaluation criteria
0	No coarse wrinkles
2	A few shallow, coarse wrinkles across the back skin area are observed occasionally (Bisset's Grade 1)
4	Shallow, coarse wrinkles across the back skin are observed on the whole surface (Bisset's Grade 2)
6	Some deep, long wrinkles across the back skin are observed (Bisset's Grade 3)

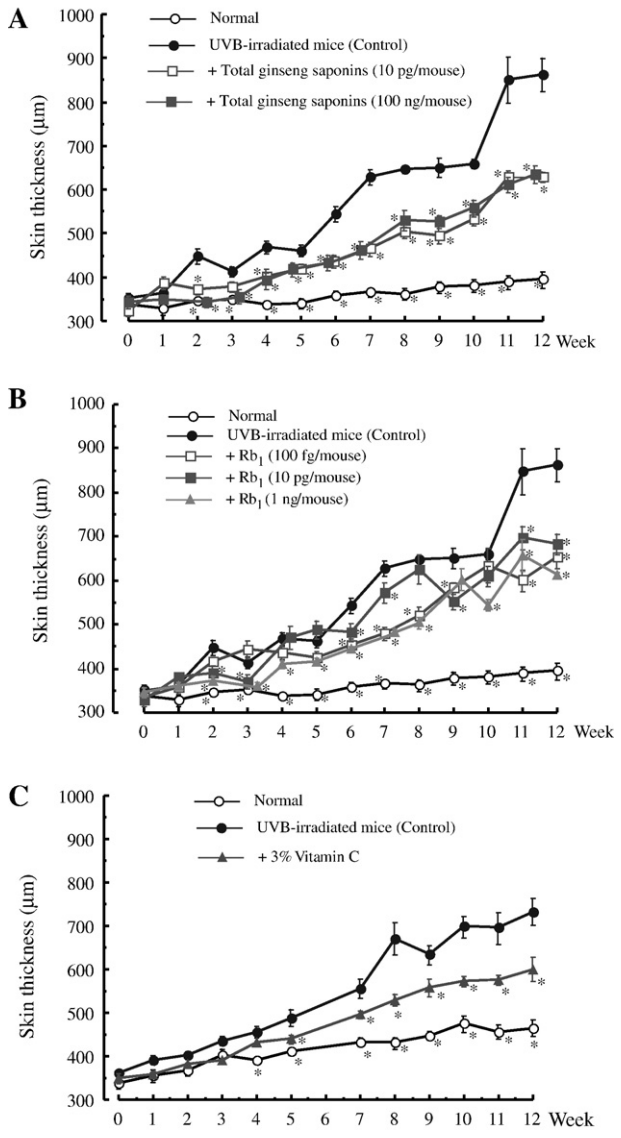

Fig. 2. Effects of total ginseng saponins, ginsenoside Rb_1, and vitamin C on skin thickness in chronic UVB-irradiated mice. Value are means±S.E.M. for 6 mice. *Significantly different from UVB-irradiated mice (control), $P<0.05$.

for 10 min at 4 °C, the TGF-β1 and total glutathione contents in the supernatant were determined using a TGF-β1-ELISA kit (R & D Systems, Minneapolis, MN, USA) and Total Glutathione Quantification kit (Dojindo Co., Kumamoto, Japan), respectively.

2.6. Measurement of epidermis and corium thickness by histological examination in UVB-irradiated skin

The dorsal skin samples (about 3 cm^2) removed at week 12 were fixed in 10% buffered formalin for at least 24 h, progressively dehydrated in solutions containing an increasing percentage of ethanol (70%, 80%, 95%, and 100%, v/v), cleared in Histoclear (AS-ONE, Tokyo, Japan), embedded in paraffin under vacuum, sectioned at 5 μm-thickness, deparaffinized, and stained with hematoxylin–eosin and Azan, respectively. After the same cross sections were selected from three plates per sample, four different microscopic fields (×200 magnification) per plate were photographed. The epidermis and corium thickness were measured from the samples stained by HE and Azan, using a Digimatic Caliper (Mitutoyo Co., Kanagawa, Japan).

2.7. Measurement of distribution of apoptosis, Ki-67, 8-OhdG, and collagen type I expression by immunohostochemical examination in UVB-irradiated skin

To determine the level of apoptotic cells, the paraffin-embedded skin sections were de-paraffinized and analyzed by the TUNEL method using an Apoptosis In Situ Detection kit (Wako Pure Chemical Co., Osaka, Japan). Four different microscopic fields (×400 magnification) per plate were photographed, and the TUNEL-positive apoptotic cells in the skin were counted. The expression levels of Ki-67 (marker of cellular proliferation) (Urruticoechea et al., 2005), 8-OHdG (marker of oxidative DNA damage) (Toyokuni et al., 1997), and collagen type I in the skin were examined by an immunoperoxidase technique using anti-mouse Ki-67, anti-8-OHdG, and anti-mouse collagen type I antibodies, respectively. The measurement of Ki-67- and 8-OHdG-positive cells was performed by the same methods as described above. To determine the distribution and structure alteration of collagen fiber,

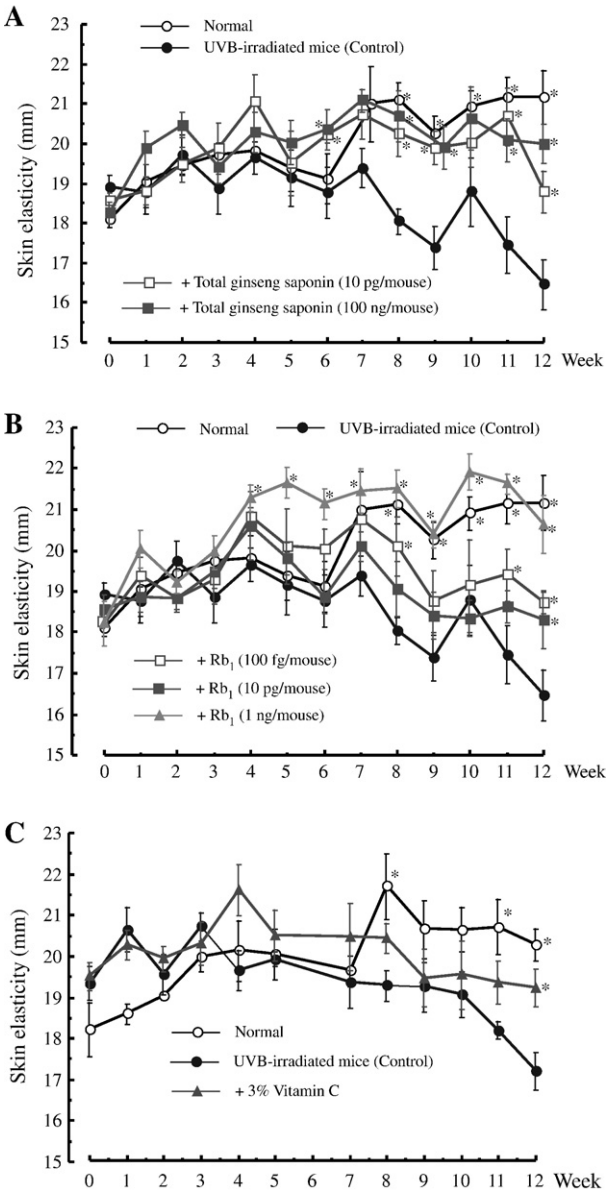

Fig. 3. Effects of total ginseng saponins, ginsenoside Rb_1, and vitamin C on skin elasticity in chronic UVB-irradiated mice. Values are means±S.E.M. for 6 mice. *Significantly different from UVB-irradiated mice (control), $P<0.05$.

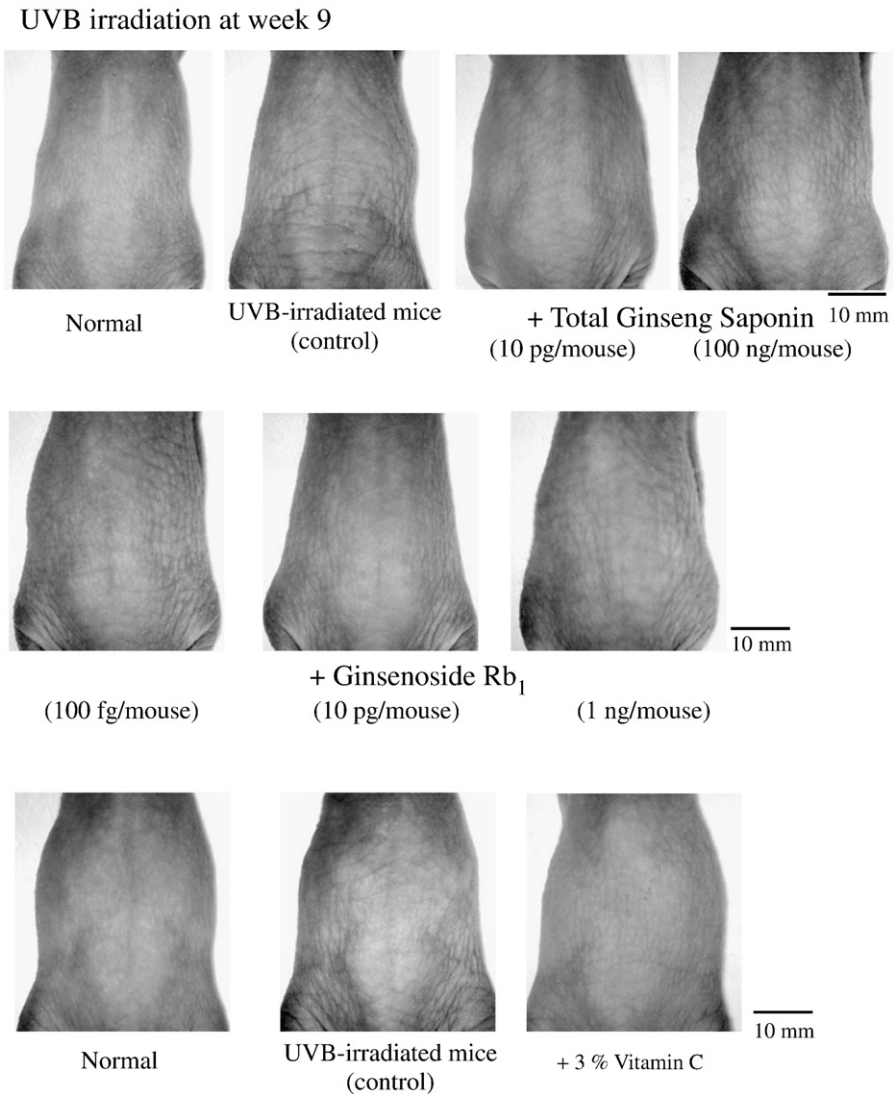

Fig. 4. Photographs showing skin wrinkles induced by chronic UVB irradiation and the effects of topically applied total ginseng saponins, ginsenoside Rb_1, and vitamin C.

the disarrangement and fragmentation of collagen fiber were evaluated by the determination of length and arrange angle to epidermis of collagen fiber, from the immunohistochemical observation stained by anti-collagen type 1 antibody.

2.8. Measurement of Bcl-2, Bax, and Bak expression levels induced by UVB irradiation in human primary keratinocytes

To examine the effects of ginsenoside on Bcl-2, Bax, and Bak expression levels after UVB irradiation in human keratinocytes, human keratinocytes (3×10^5 cells) were seeded in 100-mm culture dishes and cultured in KG-2 medium for 48 h. After reaching about 80% confluency, the medium was changed for fresh KB-2 medium and the cells were cultured overnight. The cells were irradiated with UVB (20 mJ/cm^2) and treated with the indicated amounts of ginsenoside Rb_1 for 24 h in KB-2 medium. After the cells were washed with phosphate buffered saline (PBS, pH 7.0), the cells were lysed with 400 μl of cell lysis buffer [20 mM Tris–HCl (pH 7.5) containing 150 mM NaCl, 1 mM EDTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride]. After centrifugation at 12,000 ×g for 10 min at 4 °C, the supernatant was used for the measurement of Bcl-2, Bax, and Bak protein levels. Samples (10 μg protein) were boiled for 5 min, subjected to electrophoresis in a 7.5% polyacrylamide gel, and used for western blot analysis with anti-Bcl-2, anti-Bax, anti-Bak, and anti-β-actin antibodies.

Table 2
Effects of total ginseng saponins, ginsenoside Rb_1, and vitamin C on skin wrinkles induced by UVB irradiation in hairless mice

	Wrinkle score	
	6 weeks	9 weeks
Experiment 1		
Normal mice	0±0	0±0
Vehicle-treated UVB-irradiated mice (control)	3.8±0.54	4.8±0.4
+Total ginseng saponins		
(10 pg/mouse)	1.3±0.21[a]	2.2±0.31[a]
(100 ng/mouse)	1.3±0.21[a]	2.3±0.42[a]
+Ginsenoside Rb_1		
(100 fg/mouse)	1.3±0.21[a]	4.2±0.31
(10 pg/mouse)	3.0±0.45	3.7±0.21[a]
(1 ng/mouse)	1.5±0.22[a]	4.7±0.33
Experiment 2		
Normal mice	0±0	0±0
Vehicle-treated UVB-irradiated mice (control)	2.6±0.24	2.9±0.24
+3% Vitamin C	0.6±0.35[a]	1.8±0.37[a]

Values are means±S.E.M. for 6 mice.
[a] Significantly different from UVB-irradiated mice (control), $P<0.05$.

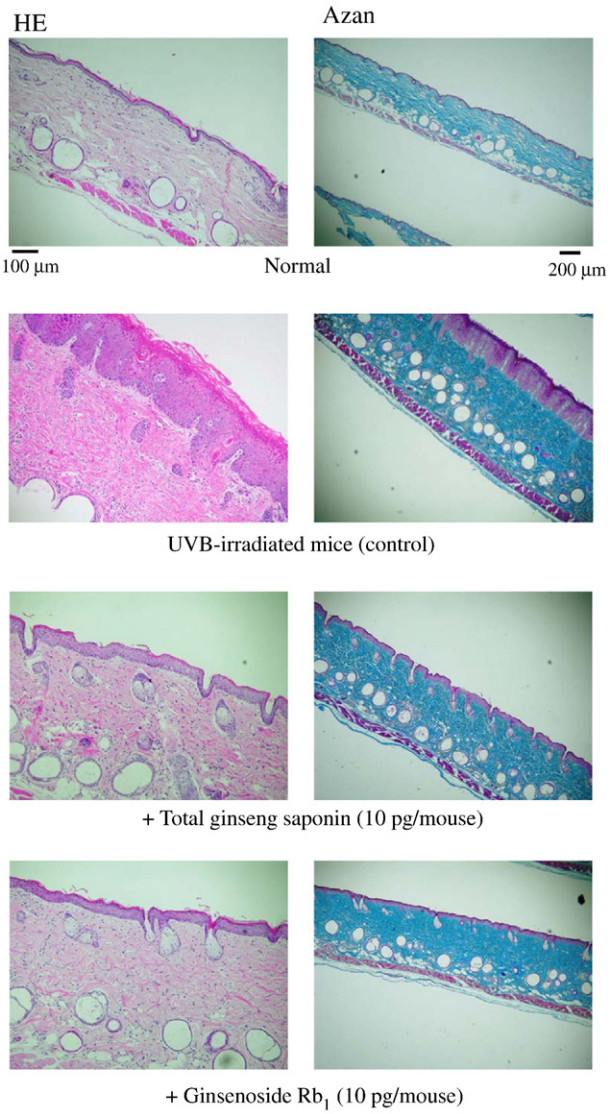

Fig. 5. Light micrographs of cells stained with hematoxylin–eosin (HE) and Azan in normal mice, vehicle-treated chronic UVB-irradiated mice (control), total ginseng saponin-treated UVB-irradiated mice, ginsenoside Rb_1-treated UVB-irradiated mice.

2.9. Statistical analysis

All values are expressed as means±S.E.M. Data were analyzed by one-way ANOVA, and then differences among means were analyzed using Dunnett's test or Tukey–Kramer's multiple comparison test. Differences were considered significantly at $P<0.05$.

3. Results

3.1. Effects of total ginseng saponins, ginsenoside Rb_1, and vitamin C on skin thickness, elasticity, and wrinkles induced by UVB irradiation

Skin thickness increased significantly during weeks 2 to 12 of UVB irradiation (Fig. 2). On the other hand, skin elasticity reduced significantly during weeks 8 to 12 of UVB irradiation (Fig. 3). The formation of wrinkles began to be observed macroscopically in the dorsal region at weeks 6 and 9 after the initiation of UVB irradiation (Fig. 4). The topical application of total ginseng saponin (10 pg or 100 ng/mouse) and ginsenoside Rb_1 (100 fg, 10 pg, or 1 ng/mouse) significantly inhibited the increase in skin thickness induced by UVB exposure during weeks 2 to 12 compared to the skin thickness of vehicle-treated UVB-irradiated mice (control) (Fig. 2A and B). The topical application of 3% vitamin C also inhibited the increase in skin thickness induced by UVB exposure during weeks 5 to 12 compared to the skin thickness of control mice (Fig. 2C). The reduction in skin elasticity induced by UVB exposure was significantly inhibited by the topical application of total ginseng saponins (10 pg or 100 ng/mouse) and ginsenoside Rb_1 (100 fg, 10 pg, or 1 ng/mouse) during weeks 6 to 12 compared to that of control mice (Fig. 3A and C). The topical application of 3% vitamin C inhibited the reduction in skin elasticity induced by UVB exposure at week 12 compared to that of control mice (Fig. 3C). Wrinkle formation induced by UVB exposure at weeks 7 and 9 was inhibited by the topical application of total ginseng saponin (10 pg or 100 ng/mouse), ginsenoside Rb_1 (100 fg, 10 pg, or 1 ng/mouse), and 3% vitamin C (Fig. 4A and B, and Table 2).

3.2. Effects of total ginseng saponins, ginsenoside Rb_1, and vitamin C on the thickness of the epidermis and extracellular matrix (ECM) of the corium in the skin of UVB-irradiated hairless mice

Based on histological observation by HE and Azan stains of dorsal skin, the thickness of the epidermis (HE stained samples) and extracellular matrix (ECM) of the corium (Azan stained samples) in the skin was significantly increased by UVB irradiation compared to that in normal mice. The topical application of total ginseng saponins (10 pg or 100 ng/mouse), ginsenoside Rb_1 (100 fg, 10 pg, or 1 ng/mouse), and 3% vitamin C significantly inhibited the increase in epidermal thickness induced by UVB exposure in the skin compared to the epidermal thickness of UVB-irradiated mice (control) (Fig. 5 and Table 3). On the other hand, total ginseng saponins, ginsenoside Rb_1, and vitamin C had no effect on the increase in ECM of the corium induced by UVB irradiation (Fig. 5 and Table 3).

3.3. Effects of ginsenoside Rb_1 on the number of apoptotic Ki-67-, and 8-OHdG-positive cells, and the length and arrange angle of collagen fiber in the skin of UVB-irradiated hairless mice

We examined the histological effects of total ginseng saponins and ginsenoside Rb_1 in the skin of UVB-irradiated hairless mice. Nuclear protein Ki-67 is a well-known marker of cellular proliferation (Urruticoechea et al., 2005), and 8-OHdG is a well-known marker of oxidative DNA damage (Toyokuni et al., 1997). It was found that the expression of Ki-67-positive cells was localized to the stratum basale (basal layer) between the epidermis and the corium, and the level of

Table 3
Effects of total ginseng saponins, ginsenoside Rb_1, and vitamin C on the thickness of the epidermis and ECM of the corium at week 12 in UVB-irradiated hairless mice

	Epidermis (μm)[a]	ECM (μm) in corium[a]
Experiment 1		
Normal mice	14.74±1.11[b]	332.51±23.18[b]
Vehicle-treated UVB-irradiated mice (control)	142.59±25.37	632.32±31.96
+Total ginseng saponins		
(10 pg/mouse)	48.66±5.04[b]	574.18±30.02
(100 ng/mouse)	54.08±3.83	494.87±23.16
+Ginsenoside Rb_1		
(100 fg/mouse)	46.00±6.26[b]	561.86±45.22
(10 pg/mouse)	49.24±4.73[b]	560.67±44.81
(1 ng/mouse)	39.84±6.26[b]	585.63±31.35
Experiment 2		
Normal mice	25.10±2.43[b]	433.00±11.00[b]
Vehicle-treated UVB-irradiated mice (control)	67.44±10.26	683.95±76.41
+3% Vitamin C	38.78±5.38[b]	603.33±36.48

[a] Values are means±S.E.M. for 6 mice.
[b] Significantly different from UVB-irradiated mice (control), $P<0.05$.

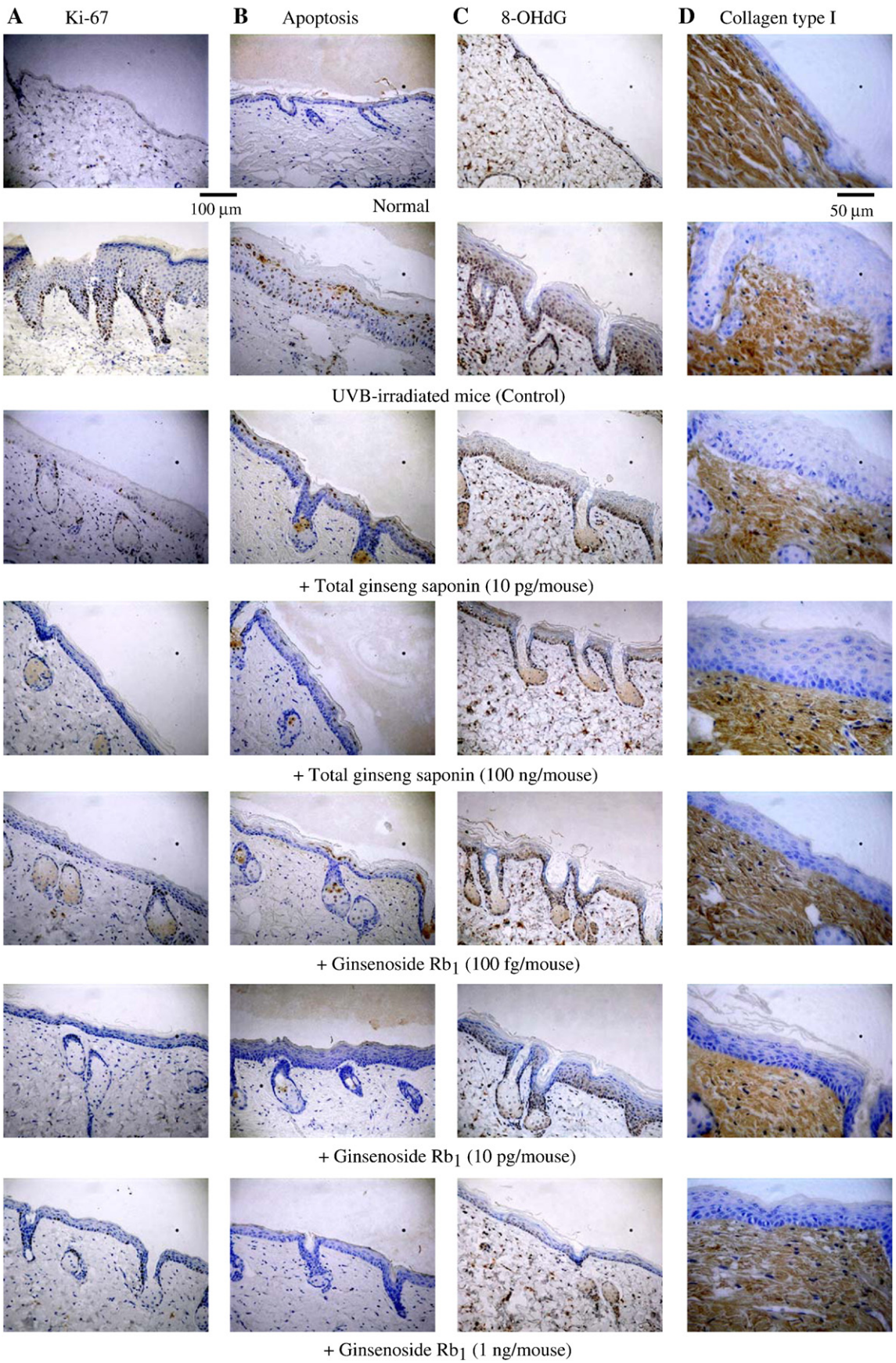

Fig. 6. Light micrographs of cells stained with anti-mouse Ki-67 rat monoclonal antibody to show keratinocyte proliferation (A), terminal deoxyribonucleotidyl transferase-mediated dUTP-digoxigenin nick end-labeling (TUNEL) method to show apoptotic cells (B), anti-8-OHdG antibody to show oxidative DNA damage (C), and anti-mouse collagen type I rabbit antibody to show the disruption of collagen type I in the dermis (D). Antibody-treated samples were stained by the immunoperoxidase technique and counterstained with hematoxylin.

Table 4
Effects of total ginseng saponins and ginsenoside Rb_1 on the numbers of apoptotic, Ki-67-, and 8-OHdG-positive cells at week 12 the skin of UVB-irradiated hairless mice

	Ki-67-positive cells (number/field)[a]	Apoptotic cells (number/field)[a]	8-OHdG-positive cells (number/field)[a]
Normal mice	9±2[b]	0±0[b]	106±7[b]
Vehicle-treated UVB-irradiated mice (control)	145±32	102±10	286±32
+Total ginseng saponins			
(10 pg/mouse)	33±7[b]	67±5[b]	148±25[b]
(100 ng/mouse)	30±18[b]	47±15[b]	170±28[b]
+Ginsenoside Rb_1			
(100 fg/mouse)	63±11[b]	19±11[b]	150±24[b]
(10 pg/mouse)	26±9[b]	11±11[b]	183±27[b]
(1 ng/mouse)	37±3[b]	9±9[b]	109±26[b]

[a] Values are means±S.E.M. for 6 mice.
[b] Significantly different from UVB irradiated mice (control), $P<0.05$.

Ki-67-positive cells was increased by UVB irradiation. The topical application of total ginseng saponins (10 pg or 100 ng/mouse) and ginsenoside Rb_1 (100 fg, 10 pg, or 1 ng/mouse) significantly reduced the increase in Ki-67-positive cell levels induced by UVB irradiation compared to the number of Ki-67-positive cells in vehicle-treated UVB-irradiated hairless mice (control) (Fig. 6A and Table 4). The occurrence of apoptotic cells was localized to the stratum granulosum of the epidermis and was increased by UVB irradiation. The increase in apoptotic cell levels induced by UVB irradiation was significantly inhibited by the topical application of total ginseng saponins (10 pg or 100 ng/mouse) and ginsenoside Rb_1 (100 fg, 10 pg, or 1 ng/mouse) compared to the number of apoptotic cells in the control mice (Fig. 6B and Table 4). The 8-OHdG-positive cells were also localized to the stratum basale and corium and their level was increased by UVB irradiation. The increase in 8-OHdG-positive cells induced by UVB irradiation was inhibited by the topical application of total ginseng saponin (10 pg or 100 ng/mouse) and ginsenoside Rb_1 (100 fg, 10 pg, or 1 ng/mouse) (Fig. 6C and Table 4). Although the range of collagen fiber in the corium was systematic in the normal mice, the range of collagen fiber was disturbed and showed fragmentation of collagen fiber as a result of UVB irradiation as shown in Fig. 6D. In fact, the arrange angle to the epidermis of collagen fiber in UVB-irradiated skin were greater than that in normal skin, conversely the length of collagen fiber in UVB-irradiated skin were shorter than that in normal skin (Table 5). The topical application of total ginseng saponins (10 pg or 100 ng/mouse) and ginsenoside Rb_1 (100 fg, 10 pg, or 1 ng/mouse) prevented the disarrangement (arrange angle to the epidermis) and fragmentation of collagen fibers induced by UVB irradiation compared to the state of collagen fibers in UVB-irradiated mice (Fig. 6D and Table 5).

Table 5
Effects of total ginseng saponins and ginsenoside Rb_1 on the length and angle to epidermis of collagen fiber at week 12 in UVB-irradiated hairless mice

	Collagen fiber	
	Length (μm)[a]	Angle (°) to epidermis[a]
Normal mice	74.31±6.12[b]	17.23±3.24[b]
Vehicle-treated UVB-irradiated mice (control)	15.08±1.86	45.64±2.37
+Total ginseng saponins		
(10 pg/mouse)	31.94±4.69[b]	27.00±2.91[b]
(100 ng/mouse)	36.68±4.48[b]	24.33±2.27[b]
+Ginsenoside Rb_1		
(100 fg/mouse)	26.69±5.42	29.67±2.08[b]
(10 pg/mouse)	34.74±7.16[b]	28.00±4.54[b]
(1 ng/mouse)	78.29±7.80[b]	25.07±3.41[b]

[a] Values are means±S.E.M. for 6 mice.
[b] Significantly different from UVB-irradiated mice (control), $P<0.05$.

Table 6
Effects of total ginseng saponins and ginsenoside Rb_1 on TGF-β1 and total glutathione contents at week 12 in skin of UVB-irradiated hairless mice

	TGF-β1 (ng/mg protein)[a]	Total glutathione (nmol/mg protein)[a]
Normal mice	0.221±0.020	86.48±10.16
Vehicle-treated UVB-irradiated mice (control)	0.248±0.014	79.93±7.03
+Total ginseng saponins		
(10 pg/mouse)	0.255±0.017	71.51±2.06
(100 ng/mouse)	0.255±0.011	72.48±2.40
+Ginsenoside Rb_1		
(100 fg/mouse)	0.248±0.006	77.07±4.10
(10 pg/mouse)	0.239±0.015	77.34±3.30
(1 ng/mouse)	0.238±0.015	72.44±3.17

[a] Values are means±S.E.M. for 6 mice.

3.4. Effects of total ginseng saponins and ginsenoside Rb_1 on TGF-β1 and total glutathione contents in the skin of UVB-irradiated hairless mice

Total ginseng saponin (10 pg and 100 ng/mouse) and ginsenoside Rb_1 (100 fg, 10 pg, and 1 ng/mouse) had no effect on TGF-β1 or total glutathione levels in UVB-irradiated hairless mice (Table 6).

3.5. Effects of total ginseng saponins and ginsenoside Rb_1 on UVB-irradiated Bcl-2, Bax and Bak expression in human keratinocytes (in vitro)

UVB (20 mJ/cm^2) irradiation slightly reduced the level of Bcl-2 expression in human primary keratinocytes. On the other hand, in this study, UVB irradiation had no effect on the Bak or Bax expression

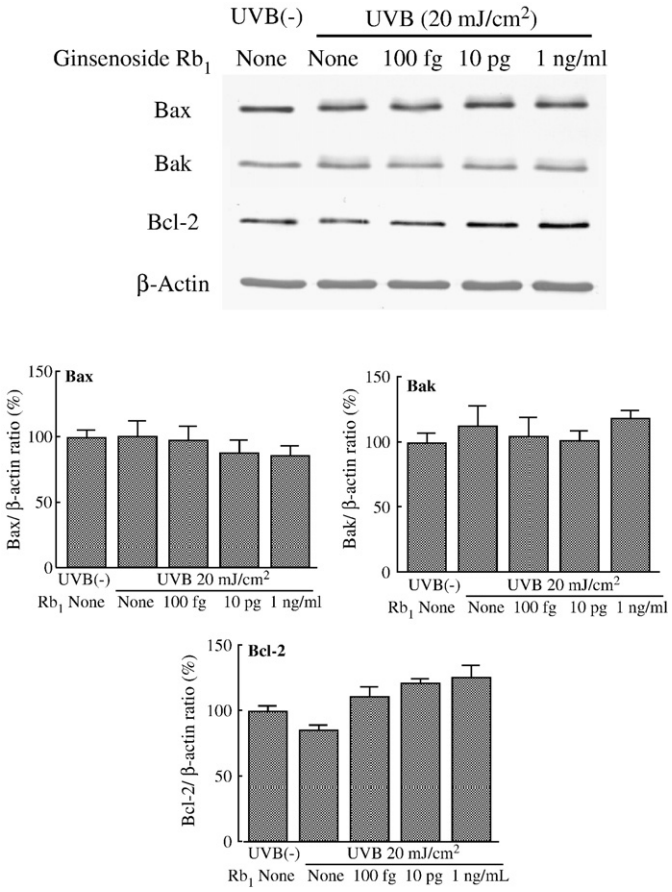

Fig. 7. Effects of ginsenoside Rb_1 on Bax, Bak, and Bcl-2 expression levels in UVB-irradiated human primary keratinocytes. Values are means±S.E.M. of 4 experiments. The relative density of the bands was normalized to β-actin.

levels (Fig. 7). Ginsenoside Rb_1 increased the Bcl-2 expression level in UVB-treated human primary keratinocytes at concentrations of 100 fg, 10 pg, and 1 ng/ml. On the other hand, ginsenoside Rb_1 had no effect on the Bak or Bax expression levels in UVB-treated human primary keratinocytes (Fig. 7).

4. Discussion

It is well-known that the symptoms of cutaneous aging, such as winkles and pigmentation, develop earlier in sun-exposed skin than in unexposed skin, a phenomenon referred to as photoaging. UVB radiation is one of the most important environmental factors because of its hazardous health effects, which include the generation of skin cancer (de Gruijl et al., 1993) suppression of the immune system (Beissert and Schwarz, 1999), and premature skin aging (Fisher et al., 1997). We reported previously that the oral administration of Red Ginseng extracts (20 or 60 mg/kg, twice daily) prevented the increase in skin pigmentation and thickness induced by acute UVB-irradiation (UVB irradiation was performed daily at a dose of 120 mJ/cm^2 for 3 consecutive days and then for 11 days every other day) in C57Bl/6J mice (Kim et al., 2008). However, the effects of total ginseng saponins and ginsenoside Rb_1 on skin photoaging such as wrinkle formation, skin thickness, and skin elasticity induced by long-term low-energy UVB exposure have not yet been demonstrated. This study found that total ginseng saponins and ginsenoside Rb_1 in total saponins inhibited the increase in skin thickness and the formation of wrinkles, and the reduction in skin elasticity induced by long-term UVB irradiation at very low doses. Furthermore, based on immunohistochemical observations, the topical application of total ginseng saponins and ginsenoside Rb_1 inhibited the increase in apoptotic, Ki-67-, and 8-OHdG-positive cells induced by long-term UVB irradiation. UVB exposure of skin cells results in several types of DNA damage such as the formation of cyclobutane pyrimidine dimers, pyrimidine pyrimidone photodimers, and 8-OHdG (Budiyanto et al., 2000; Katiyar et al., 2000; Cadet et al., 2005). UV exposure produces reactive oxygen species that can also damage DNA molecules and other lipid components ultimately leading to carcinogenesis (F'guyer et al., 2003; Bowden, 2004; Nishigori et al., 2004). Therefore, it seems likely that the reduced formation of 8-OHdG-positive and apoptotic cells in the skin may be attributed to the scavenging action of ginsenoside Rb_1. Ginsenoside Rb_1 had no effect on total glutathione levels in UVB-irradiated skin (*in vivo*) or radical scavenging action using a 1,1-diphenyl-2-picrylhydrozyl radical assay (*in vitro*) (data not shown). Therefore, the UVB-irradiated skin aging cannot be directly explained by the scavenging effect of ginsenoside Rb_1 on reactive oxygen species induced by UVB exposure. There are many reports that apoptotic stimuli such as UV radiation, tumor necrosis factor-α, Fas ligand, and chemotherapeutic drugs induce cell death by activating caspases (Cryns & Yuan, 1998). Bcl-2 is a member of the large Bcl-2 family and protects cells from apoptosis. Bcl-2 is found on the cytoplasmic face of the outer mitochondrial membrane, nuclear envelope, and endoplasmic reticulum, and other Bcl-2 family members either residue on one or more of these membranes or congregate there during apoptosis (Kaufmann et al., 2003). It has been reported that Bax and Bak appear to premeabilize the outer mitochondrial membrane, allowing the efflux of apoptogenic proteins (Green & Reed, 1998; Martinou & Green, 2001; Newmeyer & Ferguson-Miller, 2003). Bax binds to the mitochondrial membrane and induces cytochrome *c* release that subsequently activates caspase-9 and caspase-3 leading to downstream apoptotic responses (Cory & Adams, 2002). Therefore, it is suggested that the protective effect of ginsenoside Rb_1 on UVB-mediated apoptosis may be partly due to the up-regulation of Bcl-2 expression in human keratinocytes. Furthermore, the topical application of ginsenoside Rb_1 prevented the disturbance of the systematic range of collagen induced by UVB exposure in the corium. Aging of the skin is manifested as increases in skin thickness and formation of wrinkles and reduction in skin elasticity, which are fundamentally associated with reductions in the level of collagen type I, the principal component of the dermal layer of the skin. Chronic UVB irradiation induces keratinocyte proliferation and epidermal hyperplasia and decreases the production of type I procollagen, thereby leading to the loss of collagen, and consequently to increases in wrinkle formation and skin thickness, and the reduction of skin elasticity (Yaar & Gilchrest, 2007). Inomata et al. (2003) reported that matrix metalloproteinases (MMPs) increase in activity over wide areas of mouse skin during chronic UVB exposure and contribute to wrinkle formation through destruction of the basement membrane structure followed by degradation of extracellular matrix components, such as collagen fibers. In this study, the increase in Ki-67-positive cells in the stratum basale (epidermal hyperplasia and keratinocyte proliferation) induced by chronic UVB irradiation was prevented by the topical application of ginsenoside Rb_1 at low doses of 100 fg, 10 pg, and 1 ng/mouse. There are a number of reports that ginseng saponins suppress MMPs expression in various cells (Fujimoto et al., 2001; Yue et al., 2006; Jung et al., 2006; Choo et al., 2008). Lee et al. (2007) reported that Ginseng extract induced collagen (type I) synthesis through the activation of Smad signaling in human dermal fibroblast cells. Therefore, it seems likely that the protective effect of ginsenoside Rb_1 on skin photoaging induced by chronic UVB exposure may be due to the increase in collagen synthesis and/or the inhibition of MMPs expression in dermal fibroblast and the inhibition of epidermal hyperplasia. Further research is needed to clarify the mechanisms of the protective effect of ginsenoside Rb_1 on photoaging induced by chronic UVB irradiation of the skin. In this study, we could not show the dose–response effect of ginseng saponins on UVB-induced photoaging at the lower concentrations. Previously, we reported that the promotion of burn wound healing by total ginseng saponin (10 pg and 100 ng/mouse) and ginsenoside Rb_1 (100 fg, 10 pg and 1 ng/mouse) at the lower concentrations might be due to the promotion of angiogenesis (Kimura et al., 2006). In the above experiments, there are no significant differences among the low doses of ginseng saponins. Furthermore, Zhang et al. (2005) reported that intravenous infusion of ginsenoside Rb_1 at low doses (6 and 60 μg/day), prevented ischemic neouronal death through the upregulation of Bcl-x_L expression, but high doses (3 and 12 mg/day) it had no effect. Thus, the pharmacological actions of ginseng saponins are exhibited at low doses, and the dose–response effect of ginseng saponins are not shown. The reason for this is unknown; therefore, further studies are needed. Our results provide a basis for the photochemopreventive effect of ginsenoside Rb_1 at very low doses (100 fg to 1 ng/mouse) and suggest that it may be a useful agent against UVB-induced skin damage. This is the first report showing that the topical application of total ginseng saponins and ginsenoside Rb_1 prevents UVB-induced skin photoaging at very low doses.

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