**FULL COMMUNICATION**

# Royal Jelly Protects Against Ultraviolet B–Induced Photoaging in Human Skin Fibroblasts via Enhancing Collagen Production

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ABSTRACT    Royal jelly (RJ) is a honeybee product containing proteins, carbohydrates, fats, free amino acids, vitamins, and minerals. As its principal unsaturated fatty acid, RJ contains 10-hydroxy-2-decenoic acid (10-HDA), which may have antitumor and antibacterial activity and a capacity to stimulate collagen production. RJ has attracted interest in various parts of the world for its pharmacological properties. However, the effects of RJ on ultraviolet (UV)-induced photoaging of the skin have not been reported. In this study we measured the 10-HDA content of RJ by high-performance liquid chromatography and tested the effects of RJ on UVB-induced skin photoaging in normal human dermal fibroblasts. The effects of RJ and 10-HDA on UVB-induced photoaging were tested by measuring procollagen type I, transforming growth factor (TGF)-$\beta$1, and matrix metalloproteinase (MMP)-1 after UVB irradiation. The RJ contained about 0.211% 10-HDA. The UVB-irradiated human skin fibroblasts treated with RJ and 10-HDA had increased procollagen type I and TGF-$\beta$1 productions, but the level of MMP-1 was not changed. Thus RJ may potentially protect the skin from UVB-induced photoaging by enhancing collagen production.

KEY WORDS: • *10-hydroxy-2-decenoic acid* • *matrix metalloproteinase-1* • *photoaging* • *procollagen type I* • *royal jelly* • *transforming growth factor-β1*

## INTRODUCTION

SKIN AGING CAN BE DIVIDED into intrinsic and extrinsic aging. Extrinsic aging is mainly due to photodamage caused by ultraviolet (UV) irradiation. UV irradiation is a major environmental factor in acute and chronic disorders of the human skin. Chronic UV exposure is the primary cause of premature aging of the skin, so-called photoaging, which is characterized clinically by thickening, roughness, coarse wrinkles, and mottled pigmentation. Histologic changes include collagen cross-linking, excessive deposition of abnormal elastic fibers, and an increase in glycosaminoglycans.[1–5] UV light is composed of UVA (320–400 nm), UVB (280–320 nm), and UVC (100–280 nm). UVB radiation generates reactive oxygen species, which directly damage DNA and induce formation of pyrimidine dimers. The reactive oxygen species also activate signaling for DNA repair and antioxidant defense and induce expression of matrix metalloproteinases (MMPs).[3,6] The MMPs make up a family of structurally related matrix-degrading enzymes involved in tissue-destructive processes such as aging of the skin, arthritis, and tumor invasion. MMPs also degrade collagen and other extracellular matrix proteins, and MMP-1 targets collagen types I, II, and III in the skin.[7,8]

Worker-caste (nurse) honeybees produce royal jelly (RJ) through digestion of pollen in the hypopharyngeal and mandibular glands. Worker bees distribute this proteinaceous food to developing larvae and to every adult bee in the colony. Female larvae destined to become queens receive larger amounts of RJ in a critical period of development. RJ consists of proteins, carbohydrates, fats, free amino acids, vitamins (biotin, folic acid, inositol, niacin, pantothenic acid, pyridoxine, riboflavin, thiamine, and vitamin E), and minerals (copper, zinc, iron, calcium, manganese, potassium, and sodium) and significant amounts of bioactive substances such as the unsaturated fatty acid 10-hydroxy-2-decenoic acid (10-HDA).[9–11] Pharmacologically, RJ displays vasodilative, hypotensive, antitumor, antihypercholesterolemic, anti-inflammatory, and antioxidative activities. RJ also scavenges superoxide anion, 1,1-diphenyl-2-picrylhydrazyl radical, and hydroxyl radical. The major fatty acid component of RJ, 10-HDA, has been shown to have antitumor, collagen synthesis-inducing, and MMP-inhibitory activities.[12–16] Based on reports of its efficacy, RJ is used in cosmetics and as a functional food, but evidence to support this use is

weak. A recent study showed that 10-HDA induced a dose-dependent increase in collagen production in normal human dermal fibroblasts (NHDFs). The same treatment also increased the level of transforming growth factor (TGF)-$\beta$1, a factor involved in collagen production. However, the effects of RJ on UV-induced photoaging of the skin have not been reported. In this study we aimed to measure the 10-HDA content of RJ and to test the effects of RJ on UVB-induced skin photoaging in NHDFs. As end points for the effect of RJ, we measured procollagen type I and MMP-1, markers of skin photoaging, and also determined the amount of TGF-$\beta$1 in UVB-exposed and nonexposed NHDFs.

## MATERIALS AND METHODS

### Reagents

Distilled water and acetonitrile were purchased from Burdick & Jackson, SK Chemicals (Ulsan, Korea); 10-HDA, from Nacalai USA, Inc. (San Diego, CA, USA); and trifluoroacetic acid, from Alfa Aesar (Ward Hill, MA, USA). All chemicals and solvents were of high-performance liquid chromatography (HPLC) grade commercially available.

### Sample preparation

RJ collected in Korea (originating in Cheorwon, Korea) was used throughout the experiments.

### Quantitative analysis of 10-HDA from RJ by HPLC

The 10-HDA content of RJ was determined by HPLC (Agilent 1100 HPLC system, Agilent Technologies, Inc., Santa Clara, CA, USA) using a Symmetry C18 column (250×4.6 mm i.d., S-4 $\mu$m, 80 Å; YMC Co., Ltd., Kyoto, Japan). The detection wavelength was set at 210 nm. The standards and samples were separated using a linear gradient consisting of acetonitrile (A) and water (B) under the following conditions: 0–20 minutes, 30–100% A; 20–25 minutes, 100% A. The run time was 25 minutes. The flow rate was 1.0 mL/minute, and the column was maintained at 25°C. A 10 $\mu$g/mL stock solution of the standard was prepared in methanol. Each

working standard solution was made by diluting the corresponding stock solution in methanol to 0.625–10 $\mu$g/mL. The injection volume for all test samples and standards was 10 $\mu$L.

### Cell culture

NHDFs were obtained from a young healthy male donor by skin biopsy (MCTT Core, Inc., Seoul, Korea). The cells were cultured at 37°C under 5% $CO_2$ in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin (Gibco-BRL, Gaithersburg, MD, USA). For all experiments, only those cells between passages 5 and 9 were used.

### UV irradiation and treatment of cells with RJ and 10-HDA

Royal jelly and 10-HDA were prepared in dimethyl sulfoxide and stored at $-20$°C until use. NHDFs were seeded in 40-mm-diameter dishes ($1.2\times10^5$ cells per dish) in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and antibiotics. After 24 hours, the medium was removed, the monolayer was washed with warm phosphate-buffered saline, and 1 mL of phosphate-buffered saline was added to each well. The cells were then irradiated for 40 seconds with UVB (144 mJ/cm$^2$) with the plate closed. The UVB radiation was supplied by a closely spaced array of five sunlamps (model G9T5E, Sankyo Denki Co., Tokyo, Japan), which delivered uniform irradiation at a distance of 7.5 cm. The energy output of the UVB (290–320 nm, peak 312 nm) lamps was measured with a radiometer (Herbert Waldmann GmbH & Co. KG, Villingen-Schwenningen, Germany). After irradiation, the cells were washed with warm phosphate-buffered saline. Serum-free Dulbecco's modified Eagle's medium (1,980 $\mu$L) and a test treatment (20 $\mu$L) were then added to each well. The concentrations of the RJ treatment were 1, 10, and 100 $\mu$g/mL, and those of 10-HDA were 1, 10, and 100 $\mu$M. The cells and supernatants were harvested for assays following an additional 72-hour culture period. The culture medium was frozen at $-20$°C until use.

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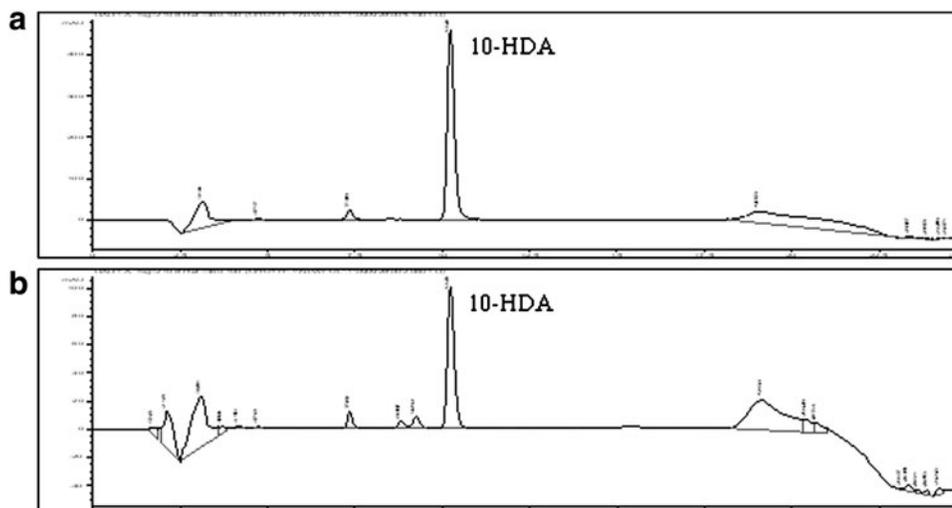


FIG. 1. High-performance liquid chromatograms of (**a**) (*E*)-10-hydroxy-2-decenoic acid (10-HDA) standard and (**b**) royal jelly.

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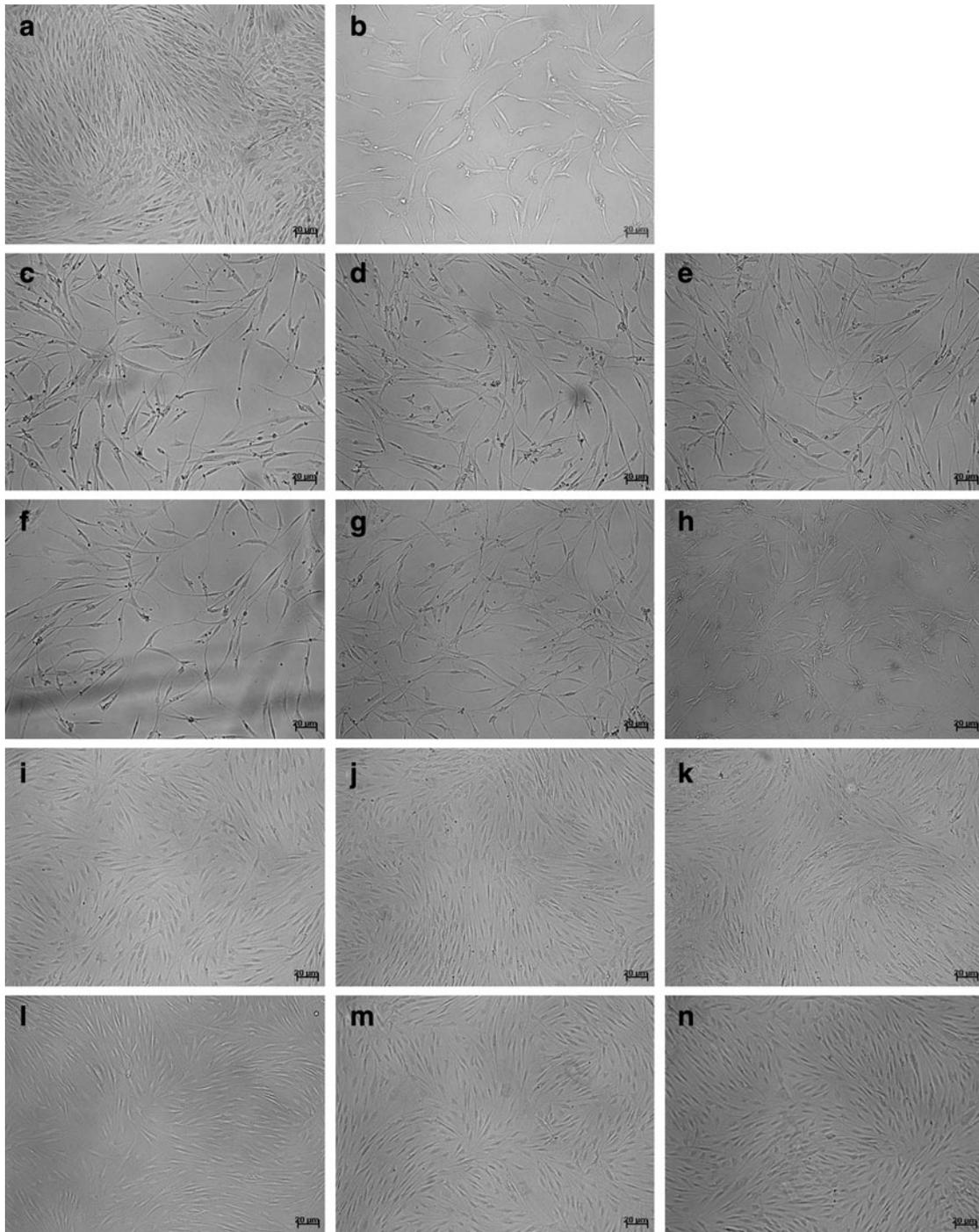
**FIG. 2.** Morphological changes in ultraviolet B–irradiated and royal jelly– or 10-HDA-treated cultured human dermal fibroblasts. The cells were visualized using microscopic imaging ($\times 100$ magnification). (**a**) Nonirradiated control cells. (**b**) Ultraviolet B–irradiated cells (144 mJ/cm$^2$). (**c–h**) Ultraviolet B–irradiated cells (144 mJ/cm$^2$) treated with (**c**) 1 $\mu$g/mL, (**d**) 10 $\mu$g/mL, or (**e**) 100 $\mu$g/mL royal jelly or (**f**) 1 $\mu M$, (**g**) 10 $\mu M$, or (**h**) 100 $\mu M$ 10-HDA. (**i–n**) Nonirradiated cells treated with (**i**) 1 $\mu$g/mL, (**j**) 10 $\mu$g/mL, or (**k**) 100 $\mu$g/mL royal jelly or (**l**) 1 $\mu M$, (**m**) 10 $\mu M$, or (**n**) 100 $\mu M$ 10-HDA.

*Morphological changes*

Morphological changes in the cultured human dermal fibroblasts were checked following an additional 72-hour culture period. The cells were visualized using a camera attached to a microscope ($\times 100$ magnification) (Olympus Corp., Tokyo).

*Procollagen type I, MMP-1, and TGF-$\beta$1 measurements*

The concentrations of type I procollagen, MMP-1, and TGF-$\beta$1 in the medium were determined using commercially available enzyme-linked immunosorbent assay kits (procollagen type I C-peptide enzyme immunoassay kit [TaKaRa Bio, Inc., Otsu, Shiga, Japan] and human total

MMP-1 kit and human TGF-$\beta$1 kit [Duoset®] [R & D Systems, Inc., Minneapolis, MN, USA]) according to the manufacturers' instructions. Each sample was analyzed in triplicate. To normalize results for procollagen type I, TGF-$\beta$1, and MMP-1 production, each marker value was divided by the corresponding cell viability.

### 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay is a commonly used, sensitive, quantitative, and reliable method to determine cell viability. Following the 72-hour post-irradiation incubation, the medium was removed, MTT (0.1 mg/mL) was added to each well, and plates were incubated at 37°C for 2 hours in a $CO_2$ atmosphere. The substrate-containing medium was removed at the end of the incubation, and 1 mL of dimethyl sulfoxide was added per well to dissolve the formazan crystals. The plates were then agitated on an orbital shaker for 30 minutes at ambient temperature, after which the absorbance of 200-$\mu$L aliquots was measured using a microplate reader (model E09090, Molecular Devices, San Francisco, CA, USA) at a wavelength of 570 nm.

### Statistical analysis

The data were expressed as mean ± SE values. Statistical comparison between different treatments was performed using a one-way analysis of variance followed by Duncan's test, and then Student's $t$ test to compare individual treatments to the controls was used for statistical analysis. Statistical significance was set at $P < .05$.

## RESULTS

### Quantitative analysis of 10-HDA from RJ

The retention time of the 10-HDA standard compound was 10.229 minutes (Fig. 1). Peaks from RJ samples were identified by comparison of retention times with those of the corresponding standards. The 10-HDA calibration curve was linear with a correlation coefficient of 0.9996. The RJ preparation contained 2.114 ± 0.0065 $\mu$g/g (0.211%) 10-HDA.

### Morphological changes in UVB-irradiated and RJ-treated cultured human dermal fibroblasts

As shown by optical microscopy, UVB irradiation and treatment with RJ and 10-HDA decreased cell growth (Fig. 2). Nonirradiated cells are shown in Figure 2a, and cells irradiated for 40 seconds are shown in Figure 2b. After UVB irradiation, cells were treated with RJ or 10-HDA at concentrations of 1, 10, and 100 $\mu$g/mL or 1, 10, and 100 $\mu M$, respectively, and incubated for 72 hours. Analysis by optical microscopy (Fig. 2c–e and f–h) revealed that UVB-irradiated cells became longer, thinner, and apoptotic; however, they showed weak dose-dependent recovery of normal morphology after treatment with RJ and 10-HDA. In the case of treatment with RJ or 10-HDA in nonexposed

cells (Fig. 2i–k and l–n), the cells displayed the dendrite pattern development in a dose-dependent manner.

### Procollagen type I expression, MMP-1 secretion, and cell viability in RJ-treated cultured human dermal fibroblasts without UVB exposure

To investigate short-term effects of RJ and 10-HDA (24 hours after treatment), procollagen type I expression and
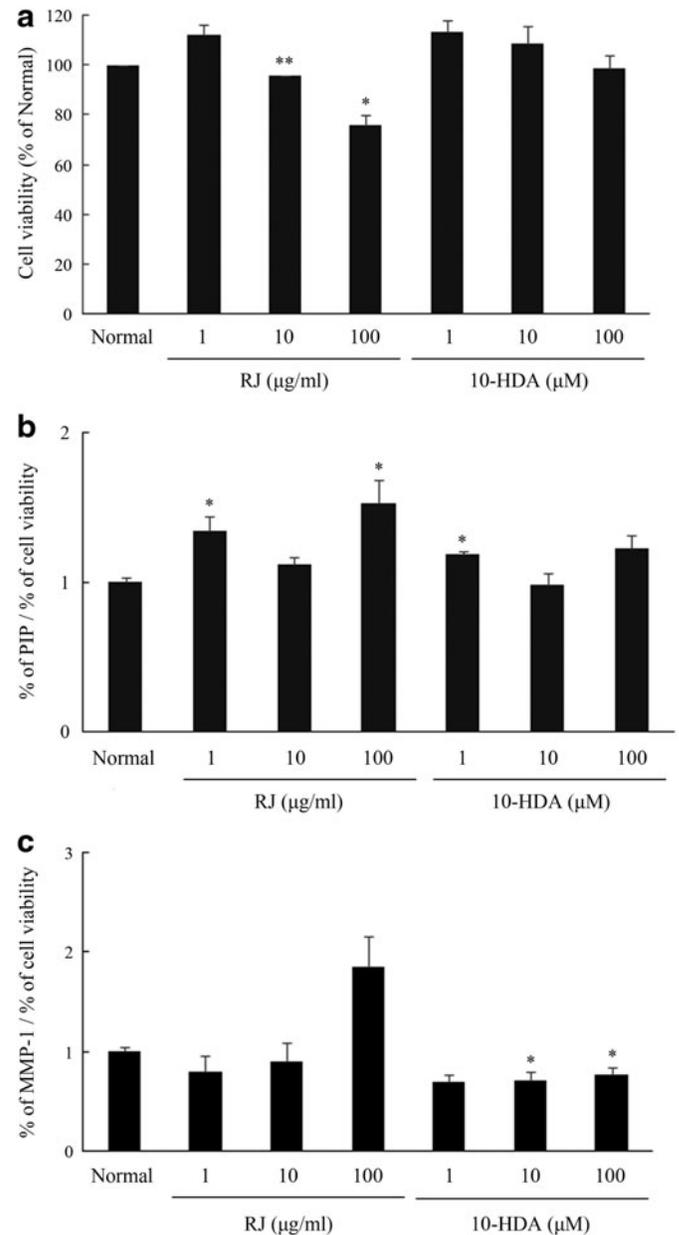


**FIG. 3.** (a) Cell viability, (b) procollagen type I production (PIP), and (c) matrix metallproteinase (MMP)-1 secretion in royal jelly (RJ)–treated cultured human dermal fibroblasts without ultraviolet B exposure. Cells were incubated in the absence or presence of RJ for 24 hours without ultraviolet B exposure. Data are mean ± SE values. Statistical significance compared with ultraviolet (−) control cells: *$P < .05$, **$P < .01$.

MMP-1 secretion were measured in cultured human dermal fibroblasts without UVB exposure. The viability of the nonirradiated control cells was considered to be 100%. At high concentration, RJ (100 μg/mL) showed cytotoxicity, whereas 10-HDA (100 μM) showed no toxicity, in the cells (Fig. 3a). MMP-1 production did not differ significantly between control and RJ-treated cells, except that cells showing cytotoxic effects at high RJ concentration (100 μg/mL) showed higher MMP-1 secretion than the others. UVB-exposed, 10-HDA-treated cells produced slightly less MMP-1 than control cells (Fig. 3c). Treatment with RJ at a low concentration (1 μg/mL) induced greater procollagen type I production than no treatment or treatment with sub-toxic concentrations of 10-HDA (Fig. 3b).

*F3* ▶

*Procollagen type I expression, MMP-1 secretion, and cell viability in UVB-irradiated and RJ-treated cultured human dermal fibroblasts*

To investigate the UVB-protective effects of RJ and 10-HDA, we measured the concentration of procollagen type I, the main type of collagen found in skin, in UVB-irradiated cultured human dermal fibroblasts. An MTT assay was used to measure cell viability (Fig. 4a), and nonirradiated control cells were considered to be 100% viable. Irradiation of the fibroblasts with UVB at $144 \, mJ/cm^2$ decreased viability to approximately 23% of that in the nonirradiated cells. Overall, RJ and 10-HDA showed weak concentration-dependent cytotoxicity. Despite toxicity at high concentrations,

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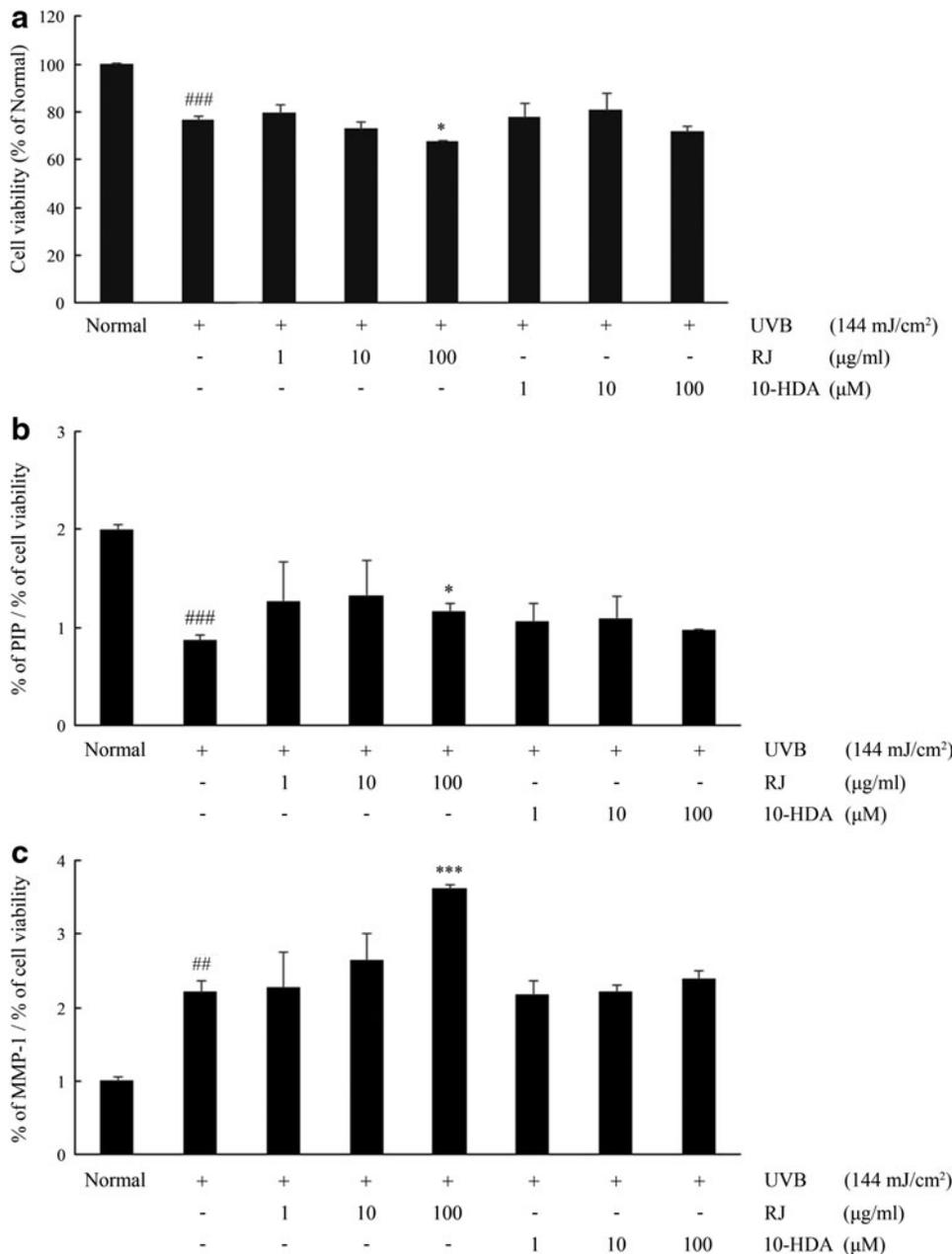


**FIG. 4.** (**a**) Cell viability, (**b**) PIP, and (**c**) MMP-1 secretion in ultraviolet (UV) B–irradiated and RJ-treated cultured human dermal fibroblasts. Cells were irradiated with UVB ($144 \, mJ/cm^2$) and then incubated in the absence or presence of RJ or 10-HDA for 72 hours. Data are mean ± SE values. Statistical significance of differences: [##]$P < .01$, [###]$P < .001$ compared with the UV (−) control; *$P < .05$, ***$P < .001$ compared with the UV (+) control.

RJ treatment increased procollagen type I production to a level higher than in UVB-irradiated cells (Fig. 4b). In other words, those cells treated with RJ and 10-HDA following UVB irradiation had a higher production of procollagen type I than those irradiated cells that were not exposed to RJ and 10-HDA. At low concentrations, RJ (1 $\mu$g/mL) and 10-HDA (1 $\mu M$) treatments increased procollagen type I secretion to levels higher than in cells not treated with these materials, yet showed no cytotoxicity.

Under oxidative stress induced by UVB, collagen degradation occurs through the action of MMP-1 and related metalloproteinases. Accordingly, we tested the effect of UVB irradiation (144 mJ/cm$^2$) on MMP-1 production (Fig. 4c). At 72 hours after the exposure, MMP-1 production was significantly increased in irradiated NHDFs compared with the nonirradiated control cells. However, the RJ and 10-HDA treatments did not detectably inhibit MMP-1 secretion in UVB-exposed NHDFs.

### Effects of RJ and 10-HDA on TGF-$\beta$1 production in UVB-irradiated NHDFs

Because TGF-$\beta$1 is well known to promote collagen production, TGF-$\beta$1 production in the culture supernatant of UVB-irradiated and RJ- or 10-HDA-treated NHDFs was determined by a Duoset enzyme-linked immunosorbent kit system. UVB exposure increased TGF-$\beta$1 by approximately 15% above that in normal cells. TGF-$\beta$1 production after RJ and 10-HDA treatments was significantly higher than that of the irradiated cells (Fig. 5). These results demonstrated that RJ and 10-HDA induced TGF-$\beta$1 production in UVB-irradiated NHDFs.

F5 ▶

## DISCUSSION

Chronic exposure of the skin to UV irradiation leads to clinical and histological changes consistent with aging, such as wrinkling, abnormal pigmentation, loss of elasticity, and reduced capacity for renewal. Biochemical markers of UV irradiation include reduced procollagen expression and increased expression of MMPs in human skin *in vitro* and *in vivo*.[1,5,17] UV-induced MMPs degrade collagen and thereby impair structural integrity of the dermis.[18] Expression of the procollagens, especially of type I procollagen, the main structural component of extracellular matrix, significantly decreases within 24 hours after a single UV exposure.[1] Biomaterials containing bioactive compounds with anti-photoaging activities are needed for both medical and cosmetic applications.

Most strategies to prevent photoaging focus on decreasing MMP production and increasing that of procollagen. This led us to investigate the effects of UVB radiation and RJ treatment on type I procollagen and MMP-1 in NHDFs. RJ is studied and used for its antioxidative, wound healing, anti-inflammatory, and antitumor activities.[12–14,19] RJ and 10-HDA are reported to promote collagen production in ascorbic acid 2-*O*-α-glucoside-treated fibroblasts by inducing TGF-$\beta$1 production.[15,20] In the present study, RJ decreased cell viability, increased collagen level, and did not alter MMP-1 production, whereas 10-HDA did not alter cell viability, increased collagen level, and decreased MMP-1 in nonirradiated cells (Fig. 3). We also showed that RJ at a nontoxic concentration (1–10 $\mu$g/mL) (Fig. 4a) significantly increased type I procollagen and TGF-$\beta$1 productions in UVB-exposed human skin fibroblasts *in vitro* (Figs. 4b and 5), whereas MMP-1 production was not changed (Fig. 4c). The TGF-$\beta$ family is well known to modulate type I collagen gene expression in fibroblasts.[21] These results demonstrated that RJ treatment up-regulated the level of type I procollagen by stimulating TGF-$\beta$1 production in UVB-induced NHDFs. Although 10-HDA, a major fatty acid component of RJ, is reported to increase collagen production, we found that 10-HDA at noncytotoxic concentrations (1–100 $\mu M$) was less effective than RJ. Because those previous reports applied 10-HDA at higher concentrations (0.5–1.5 m$M$), it seems plausible that increased production of type I procollagen by RJ represents the synergic interactions of other RJ components with or independently from 10-HDA. RJ contains various fatty acids, such as 10-hydroxydecanoic acid, 3-hydroxydecanoic acid, and 2-decenoic acid,[22,23] which are similar to 10-HDA in chemical structure, and some of these are reported to increase collagen production.[15] Although we did not specifically identify fatty acids other
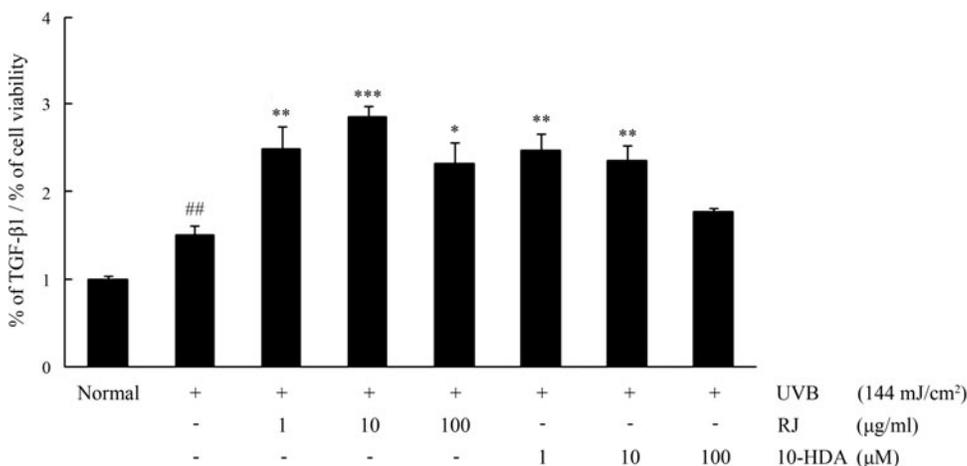


**FIG. 5.** Transforming growth factor (TGF)-$\beta$1 production by cells exposed to UVB and treated with RJ or 10-HDA. Cells were irradiated with UVB (144 mJ/cm$^2$) and then incubated in the absence or presence of RJ or 10-HDA for 72 hours. Data are mean $\pm$ SE values. Statistical significance of differences: $^{##}P < .01$ compared with the UV ($-$) control; $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$ compared with the UV ($+$) control.

than 10-HDA in our HPLC analysis, we recognize that the increases in type I procollagen observed in the RJ-treated cells might be attributed to synergistic effects of these RJ components.

MMP-1 (collagenase) plays a prominent role in collagen degradation in human dermal fibroblasts following UVB exposure.[24] In our study, RJ and 10-HDA did not significantly inhibit MMP-1 production in UVB-exposed NHDFs. Evidence suggests that in rheumatoid arthritis, 10-HDA suppresses MMP-1 and MMP-3 activities in synovial fibroblasts (which mediate joint destruction), potentially through inhibition of p38 and c-Jun N-terminal kinase–activator protein-1 signaling pathways.[16] However, these studies applied high concentrations (0.5–2 m$M$) of 10-HDA, higher than the 10-HDA concentration in the RJ extract that promoted collagen production in our experiments. Almost no mechanisms were explored in this study, so we will explore the mechanisms of these effects of RJ in greater depth in future studies.

In conclusion, this study revealed that low concentrations of RJ increased production of type I procollagen and TGF-$\beta$1 but did not influence MMP-1 secretion in NHDFs exposed to UVB. At 1 $\mu$g/mL RJ and at 1 $\mu M$ 10-HDA had no notable effect on type I procollagen production in the UVB-exposed cells. Our study supports the use of RJ for protecting skin from photoaging by promoting collagen production via up-regulation of TGF-$\beta$1 expression following UVB exposure.

## ACKNOWLEDGMENTS

## AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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