An *Ocimum basilicum* Extract Containing Rosmarinic Acid Restores the Disruption of Collagen Fibers Caused by Repetitive UVA Irradiation of Dermal Fibroblasts

Madoka Yoshikawa, Yuri Okano, and Hitoshi Masaki*

Tokyo University of Technology, 1404-1 Katakura-machi, Hachioji-shi, 192-0982 Tokyo, JAPAN

Abstract: Photoaged skin is characterized by the appearance of pigmented spots such as solar lentigos, deep wrinkles and sags, and progresses due to chronic sun exposure. Among the wavelengths of sunlight, UVA is responsible for the appearance of wrinkles and sags that originate from structural alterations in the dermis of photoaged skin such as the depletion of collagen fibers. Thus, improving and restoring collagen fibers is an effective approach to reduce skin photoaging and maintain a youthful appearance. This study was conducted to evaluate the potential of an extract of *Ocimum basilicum* (OC), which contains rosmarinic acid (RA), as an anti-photoaging material focusing on the capacity to restore collagen fibers that are disrupted due to intracellular oxidative stress. In spite of their relatively low capacities for chemical scavenging of reactive oxygen species (ROS), both OC and RA showed efficient removal of biological oxidative stress by reducing levels of intracellular ROS and carbonylated proteins (CPs) in fibroblasts following exposure to single or repetitive UVA irradiations. Fibroblasts irradiated with repetitive UVA as a model for chronic sun-exposed cells showed significant increases in matrix metalloproteinase-1 and decreases in type I collagen synthesis and formed reduced numbers of collagen fibers. Since both OC and RA restored the adverse phenomena caused by repetitive UVA irradiation, we conclude that OC containing RA is an effective anti-photoaging material.

Key words: UVA, collagen fiber, MMP-1, fibroblast

1 Introduction

It is well known that excessive UV exposure injures the skin as acute reactions such as causing inflammation and subsequent increases in wrinkles and sags. Although damage caused by UVB is limited to specific regions of the epidermis due to its limited penetration depth into the skin, UVA affects the homeostasis of the dermal matrix, including the cellular functions of fibroblasts, due to its deeper penetration into the dermis¹⁾. Thus, chronic UVA exposure of the skin is responsible for causing wrinkles and sags, which are typical symptoms of skin aging termed photoaging. The structural alterations of collagen fibers and elastic fibers that are caused by chronic UVA exposure cause fragility in the dermal matrix and result in photoaging of the skin^{2,3)}. In photoaged skin, collagen fibers, which are major fibers in the dermis, are depleted in the papillary dermis due to an imbalance of their synthesis and degrada-

tion^{4,5)}. Matrix metalloproteinases (MMPs) are responsible for the degradation of collagen molecules. Among MMPs, MMP-1, which decomposes fibrous collagen initially, is an important enzyme of collagen degradation, and is present at high levels in UV exposed skin⁶⁾. These facts indicate the importance of maintaining collagen fiber structure and eliminating the harmful actions of chronic UVA to prevent wrinkling and sagging of the skin.

The level of MMP-1 in sun-exposed skin has been well demonstrated to be regulated by reactive oxygen species (ROS)⁷⁾. In general, it is also well recognized that UVA is a potent intracellular generator of ROS. UVA generates singlet oxygen (¹O₂) through a photosensitizing reaction⁸⁾ and also superoxide anion radicals ($\cdot\text{O}_2^-$) through the activation of NADPH oxidase⁹⁾. Meanwhile, carbonylated proteins (CPs) are present at a higher frequency in the upper dermis of the sun-exposed skin of elderly subjects.

*Correspondence to: Hitoshi Masaki, Tokyo University of Technology, 1404-1 Katakura-machi, Hachioji-shi, 192-0982 Tokyo, JAPAN
E-mail: masaki@stf.teu.ac.jp

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CPs are synthesized by reactions between amino groups in proteins and aldehyde compounds yielded by lipid peroxidation initiated by ROS. Thus, CPs are regarded as a footprint of higher oxidation levels. Furthermore, our recent study reported that CPs enhance the biosynthesis of MMP-1 through the elevation of intracellular ROS¹⁰⁾. These facts indicate the importance of reducing levels of ROS in order to ameliorate the deleterious changes of collagen mediated by UVA. Furthermore, it is also important to evaluate the efficacy of materials using fibroblasts exposed to repetitive UVA as a model for fibroblasts in photoaged skin, because photoaged skin has changes in appearance caused by chronic UV exposure.

Ocimum basilicum (OC), which contains rosmarinic acid (RA) as a major component, is generally known as sweet basil. OC has been utilized as a traditional medicine, and recent studies have reported its efficacy as an antioxidant¹¹⁾. Furthermore, RA suppresses levels of ROS in HaCaT keratinocytes that are elevated by UVB through the activation of Nrf2 signals¹²⁾. These facts suggest that OC containing RA would have a potent capability to improve or prevent skin photoaging by restoring the cellular functions of fibroblasts altered by chronic UVA exposure.

Thus, the aim of this study was to clarify the potential of OC and RA as anti-photoaging materials focusing on the restoration of the formation of collagen fibers affected by repetitive UVA irradiation via the reduction of intracellular ROS levels.

2 Materials and Methods

2.1 Materials

The *Ocimum basilicum* extract (OC) which was extracted with 50% ethanol aqueous solution, was a gift from ABResearch srl Via dell'Impresa (Brendola (VI) Italy). Tris (hydroxymethyl)aminomethane, hydrochloric acid, H₂O₂, L-ascorbic acid, DL- α tocopherol, paraformaldehyde, phosphate buffer solution (PBS), sodium azide (NaN₃) and xanthine oxidase (XOD) were purchased from Nacalai tesque (Kyoto, Japan). RA, iron(II) perchlorate (Fe(ClO₄)₂), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and hypoxanthine (HPX) were obtained from FUJIFILM Wako Pure Chemical Corp. (Osaka, Japan). 5,5-Dimethyl-1-pyrroline N-oxide (DMPO), *tert*-butyl hydroperoxide (*t*-BuOOH), diethylenetriamine-N,N,N',N''-pentaacetic acid (DTPA) and 2,2,6,6-tetramethyl-4-piperidone hydrochloride (TMPD) were purchased from Tokyo Chemical Industry (Tokyo, Japan). 2-Morpholinoethanesulfonic acid, monohydrate (MES) was obtained from Dojindo (Kumamoto, Japan). Hematoporphyrin (HP) was obtained from MP Biomedicals (CA, USA). Peroxidase and ReverTra Ace qPCR Master Mix were obtained from TOYOBO (Osaka, Japan). Triton X-100 reduced and bovine serum albumin

(BSA) were obtained from Sigma-Aldrich (MO, USA). H₂DCFDA was obtained from Merck (Darmstadt, Germany). The BCA protein assay kit was obtained from Thermo Fisher Scientific (MA, USA). Fluorescein-5-thiosemicarbazine (FTSC) and Hoechst 33342 were obtained from Invitrogen Corp. (CA, USA). Anti-MMP-1 antibody (goat polyclonal) was obtained from Gene Tex (CA, USA). Anti-collagen type I antibody conjugated to biotin (rabbit polyclonal) was obtained from Rockland Scientific (B.C., Canada). Anti-goat IgG antibody conjugated to HRP and streptavidin conjugated to HRP were obtained from R&D Systems (MN, USA). Ez WestBlue was obtained from ATTO (Tokyo, Japan). Anti-rabbit IgG (H + L), F(ab')₂ fragment (Alexa Fluor 594 conjugate) was obtained from Cell Signaling (MA, USA).

2.2 Chemical scavenging capability of OC and RA against superoxide anion radicals ($\cdot\text{O}_2^-$), hydroxyl radicals ($\cdot\text{OH}$), lipid peroxyl radicals (LOO $\cdot$) and singlet oxygen ($^1\text{O}_2$)

Scavenging against ROS was measured by the ESR spin-trapping method using a RFR-30 spectrometer (Radical Research, Tokyo, Japan).

Scavenging against $\cdot\text{O}_2^-$ was carried out using a mixture of XOD (0.19 U/mL) and HPX (0.07 mM) as a source of $\cdot\text{O}_2^-$, and DMPO as a spin trapping agent. At 1 min after mixing 0.1 M phosphate buffer (pH 7.4) containing 70 μM HPX, 7 μM DTPA and 90 mM DMPO with 0.19 U/mL XOD, monitoring of the ESR spectrum was started using the ESR conditions detailed below.

Scavenging against $\cdot\text{OH}$ was carried out using H₂O₂ and Fe(ClO₄)₂ as a source of $\cdot\text{OH}$ and DMPO as a spin trapping agent. At 1 min after mixing deionized H₂O containing 40 μM Fe(ClO₄)₂, 1.18 mM DTPA, 90 mM DMPO and 160 μM H₂O₂, monitoring of the ESR spectrum was started using the ESR conditions detailed below.

Scavenging against LOO $\cdot$ was carried out using *t*-BuOOH and Fe(ClO₄)₂ as a source of LOO $\cdot$ and DMPO as a spin trapping agent. At 1 min after mixing deionized H₂O containing 480 μM Fe(ClO₄)₂, 1.18 mM DTPA, 90 mM DMPO and 1.9 mM *t*-BuOOH, monitoring of the ESR spectrum was started using the ESR conditions detailed below.

Scavenging against $^1\text{O}_2$ was measured using TMPD as a spin trapping reagent and HP as a photosensitizer to generate $^1\text{O}_2$ under UVA irradiation. After mixing 25 mM TMPD, 0.1 mM HP and sample in 0.1 M Tris/HCl (pH 8.5), the mixture was transferred into a glass capillary set in a cavity of ESR. After UVA irradiation for 45 s (3.5 J/cm²), monitoring of the ESR spectrum was started using the following ESR conditions.

ESR spectra were measured with the following conditions; output power: 4.00 mW, scanning field: 336.5 mT $\pm$ 5.0 mT, modulation frequency: 0.20 mT, response time: 0.03 s. In all measurements, the intensity of Mn(II) doped in

MnO was monitored as an external standard. Each ROS intensity was expressed as a value in which the signal intensity originating from each ROS was normalized with the signal intensity of Mn^{2+} . The scavenging capability against each type of ROS is expressed as an IC_{50} , which is the 50% scavenging concentration. IC_{50} 's of NaN_3 (for $^1\text{O}_2$), *L*-ascorbic acid (for $\cdot\text{O}_2^-$ and $\cdot\text{OH}$) and DL- α -tocopherol (for $\text{LOO}\cdot$) were measured as a positive control for each type of ROS.

2.3 Cell culture

Normal human dermal fibroblasts (NHDFs) (Kurabo, Osaka, Japan) were cultured in Dulbecco's modified Eagle medium (DMEM) (Nissui, Tokyo, Japan), supplemented with 5% fetal bovine serum (FBS) (Biowest, Nuaille, France) and incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

2.4 UVA irradiation

UVA irradiation was carried out using a FL20/BLB fluorescent lamp (Toshiba, Tokyo, Japan), which has a radiation peak at 352 nm, as the UVA light source. The irradiation energy was measured with an UVP UVX Radiometer: UVX-25 Sensor (Fisher Scientific, CA, USA). Cells were irradiated with UVA in Hanks' buffered salt solution containing Mg^{2+} and Ca^{2+} (HBSS).

2.5 Biological anti-oxidative effects of OC and RA in NHDFs irradiated by UVA

NHDFs were seeded at a density of 2×10^4 cells/well in 96 well plates and then were loaded with 20 μM H_2DCFDA for 30 min. Cells irradiated with UVA at a dose of 10 J/cm^2 were incubated in HBSS in the absence or presence of OC or RA for 6 h. After washing with HBSS, the fluorescence intensity (F.I.) (Ex: 485 nm, Em: 530 nm) of cells was measured in order to determine intracellular ROS levels. Protein contents were quantified with the BCA protein assay kit after lysing with PBS (phosphate-buffered saline without Ca^{2+} and Mg^{2+}) containing 0.5% Triton X-100. Intracellular ROS levels were calculated as F.I./ μg protein and are expressed as a fold change against the intracellular ROS level of untreated sham-UVA irradiated NHDFs. Levels of CPs in cells were examined as follows: NHDFs were exposed to UVA at a dose of 10 J/cm^2 once a day for 1 d or for 3 successive days. NHDFs were cultured in DMEM containing 5% FBS in the presence or absence of OC or RA for 24 h after each UVA irradiation. At 24 h after the last UVA irradiation, intracellular CPs were fluorescence-labeled with 20 μM FTSC in 0.1 M MES Na buffer (pH 5.5) for 1 h in the dark. Nuclei of cells were stained with 2 μM Hoechst 33342. The F.I. originating from CPs (Ex: 465 nm Em: 535 nm) and from nuclei (Ex: 350 nm; Em: 461 nm) was measured with a SpectraMax Gemini EM Microplate Reader (Molecular Devices, CA, USA). Intracellular CPs

levels were calculated as F.I. derived from FTSC labeling/ F.I. derived from Hoechst 33342, and are expressed as fold change normalized with intracellular CPs levels of untreated sham-UVA irradiated NHDFs. In addition, fluorescence images were obtained using a Floid Cell Imaging Station (Life Technology, CA, USA).

2.6 Real-time RT PCR analysis

NHDFs were irradiated with UVA at a dose of 10 J/cm^2 once a day for 3 successive days. NHDFs were cultured in DMEM containing 5% FBS in the presence or absence of OC or RA for 24 h after each UVA irradiation. At 24 h after the last UVA irradiation, total RNAs were extracted using a RNeasy Mini kit (Qiagen, CA, USA) according to the manufacturer's protocol. Total RNAs were then used for single-stranded cDNA synthesis using ReverTra Ace qPCR RT Master Mix. Quantitative real-time PCR (qPCR) was performed using the Kapa SYBR FAST with an Eco Real-Time PCR System (Illumina, CA, USA). The following primer sets for COL1A1, MMP-1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), as a housekeeping gene, were used: COL1A1 forward, 5'-CCCGGGTTTCAGAGCAACTTC-3', and reverse, 5'-TCCACA TGCTTTATTC-CAGCAATC-3', MMP-1 forward, 5'-AAGGTGGACCAAC A A T T T C A G A - 3', and reverse, 5'-TGAAGGTGTAGCTAGGGTACATCAA-3', GAPDH forward, 5'-GCACCGTAAGGCTGAGAAC-3', and reverse, 5'-TGGTGAAGACGCCAGTGGA-3'.

2.7 Western blotting

Western blotting (WB) of MMP-1 was performed to determine levels of protein expression. NHDFs were exposed to UVA at a dose of 10 J/cm^2 once a day for 3 successive days. NHDFs were cultured in DMEM containing 5% FBS in the presence or absence of OC or RA for 24 h after each UVA irradiation. At 48 h after the last UVA irradiation, supernatants of cells were collected and were mixed with equivalent volumes of sample buffer solution (FUJIFILM Wako Pure Chemical, Osaka, Japan). Ten μL of each mixture were subjected to 10% gel SDS-PAGE and were blotted to PVDF membranes (Bio Rad, CA, USA). After blocking with 1% BSA, the membranes were incubated with anti-MMP-1 antibody (1:1000). After washing, the membranes were further incubated with anti-goat IgG antibody conjugated to HRP (1:1000). After washing, MMP-1 was visualized with Ez WestBlue and was quantified using Image J.

2.8 ELISA assay

Type I collagen protein levels in the supernatants were measured using an ELISA assay as follows: The supernatants were coated on the bottom of each well in ELISA assay plates (H-plate, Sumitomo Bakelite, Tokyo, Japan) at 4°C overnight. After blocking with 1% BSA, the plates were incubated with anti-collagen I antibody conjugated to

biotin(1:5000) at 4°C overnight and were subsequently incubated with streptavidin-HRP(1:500) at room temperature for 1 h. After washing, the plates were reacted with 0.5 mg/mL ABTS in 0.1 mM sodium citrate buffer (pH 4.0) in the presence of a small amount of 30% H₂O₂ at 37°C for 20 min. Type I collagen was quantified by measuring the absorbance at 405 nm with a Multiskan FC (MA, USA). Protein contents in cells were quantified with a BCA protein assay kit after lysing with PBS containing 0.5% Triton X-100. Amounts of type I collagen protein are expressed as pg type I collagen/μg protein.

2.9 Immunofluorescence staining of collagen fibers

NHDFs were irradiated with UVA at a dose of 10 J/cm² once a day for 3 successive days. NHDFs were cultured in DMEM containing 5% FBS in the presence or absence of OC or RA for 24 h after each UVA irradiation. After the final UVA irradiation, the medium was changed to fresh DMEM supplemented with 5% FBS for 2 w. Cells were fixed with 4% paraformaldehyde for 15 min at room temperature. After blocking with 1% BSA, cells were incubated with anti-type I collagen antibody(1:500) at 4°C overnight and were then further incubated with anti-rabbit IgG (H+L) antibody-conjugated to Alexa Fluor 594(1:1000). Nuclei were stained with 2 μM Hoechst 33342. Images of fibers were obtained using a Fliod Cell Imaging Station and fiber formation was determined and is expressed as the percentage of fiber area in the whole image using corneocytometry software (CIEL, Tokyo, Japan).

2.10 Statistics

All data are expressed as means ± standard deviations. Comparisons between two groups were performed using paired t tests. A *p*-value of less than 0.05 is defined to be statistically significant.

3 Results

3.1 Chemical scavenging properties of OC and RA against ROS

In order to clarify the scavenging properties of OC and RA against ROS, IC₅₀ values for OC and RA to scavenge •O₂⁻, •OH, LOO• and ¹O₂ were examined using the ESR spin

trapping method. The results are shown in Table 1. Both OC and RA scavenged every type of ROS tested, however they showed lower scavenging properties against all types of ROS except LOO• compared with ascorbic acid, DL-α-tocopherol and NaN₃ used as positive controls.

3.2 Biological effects of OC and RA to reduce intracellular ROS and CP accumulation after UVA irradiation

UVA caused oxidative stress intracellularly by increasing levels of CPs following the elevation of intracellular ROS. Treatment with OC or RA immediately after UVA irradiation significantly reduced levels of intracellular ROS (Fig. 1a) and decreased levels of intracellular CPs (Fig. 1b, c), which are footprints of the effects of ROS. These results indicated that treatment with OC or RA after UVA exposure alleviated intracellular oxidative stress. Furthermore, the effects of OC and RA on the accumulation of intracellular CPs were examined using repetitively UVA irradiated fibroblasts as a model of chronic UVA exposure of the skin. Repetitive UVA irradiation of NHDFs once a day for 3 successive days resulted in the accumulation of CPs intracellularly, but NHDFs treated with OC or RA immediately after each UVA irradiation had lower levels of intracellular CPs (Fig. 2). These results indicated that OC and RA reduced intracellular oxidative stress initiated by UVA irradiation. This suggested that RA is an active substance in OC, because it showed similar effects to OC despite its lower concentration than in OC.

3.3 Effects of OC and RA on the synthesis and degradation of collagen

The restoration effects of OC and RA on the imbalance of the synthesis and degradation of collagen caused by repetitive UVA irradiation were examined focusing on the behaviors of type I collagen and MMP-1. Repetitive UVA irradiation changed the status of MMP-1 and type I collagen by increasing levels of MMP-1 (Fig. 3) and decreasing levels of type I collagen (Fig. 4) at both the mRNA and protein levels. Although repetitive UVA irradiation increased the protein expression level of pro-MMP-1, the active form of MMP-1 was not detected. OC and RA caused a significant suppression of MMP-1 stimulated by repetitive UVA irradiation (Fig. 3). On the other hand, both OC and RA gave conflicting results on mRNA expression and protein synthesis

Table 1

	•O ₂ ⁻				•OH				LOO•				¹ O ₂			
	IC ₅₀ (μg/mL)				IC ₅₀ (μg/mL)				IC ₅₀ (μg/mL)				IC ₅₀ (μg/mL)			
	Mean	± S.D.	p ¹	p ²	Mean	± S.D.	p ¹	p ²	Mean	± S.D.	p ¹	p ²	Mean	± S.D.	p ¹	p ²
OC	873.6	± 92.7	***	*	285.5	± 8.1	***	***	213.0	± 16.0	***	***	273.1	± 15.0	**	N.D.
RA	650.5	± 83.7	***		245.4	± 4.6	***		110.5	± 7.5	***		181.3	± 14.9	**	
<i>L</i> -ascorbic acid	79.6	± 49.0			69.8	± 0.8										
DL-α-tocopherol									803.6	± 40.5						
NaN ₃													179.4	± 11.7

Fig. 1 Suppressive effects of OC and RA on intracellular levels of ROS and CPs elevated by a single UVA irradiation. (a) After loading with H_2DCFDA , NHDFs were irradiated with UVA at a dose of $10 J/cm^2$ and were then cultured in the presence or absence of OC or RA for 6 h. Intracellular ROS levels were determined by measuring fluorescence intensity (F.I.), and were calculated by dividing F.I. by the protein content of cells (F.I./μg protein). Intracellular ROS levels are expressed as fold changes normalized with intracellular ROS levels of untreated sham-UVA irradiated NHDFs. Data are expressed as means $\pm$ standard deviations (OC: $n = 3$, RA: $n = 4$). UVA irradiation is abbreviated as UVA R. □; sham-UVA R, ■; UVA R. (b) After UVA irradiation, cells were cultured for 24 h in the presence or absence of OC or RA. Intracellular CPs levels were quantified by measuring F.I. after fluorescence labeling with FTSC, and were calculated as value of the F.I. divided by the F.I. of nuclei stained with Hoechst 33342. Intracellular CPs levels are expressed as fold changes normalized with intracellular CPs of untreated sham-UVA irradiated NHDFs. Data are expressed as means $\pm$ standard deviations ($n = 4$). □; sham-UVA R, ■; UVA R. (c) Representative images of intracellular CPs. Scale bars: 100 μm. Significance; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Fig. 2 Suppressive effects of OC and RA against intracellular CPs in NHDFs exposed to repetitive UVA irradiation. NHDFs were irradiated with UVA at a dose of 10 J/cm^2 once a day for 3 successive days. Cells were cultured in the presence or absence of OC (250 μg/mL) or RA (62.5 μg/mL) after each UVA irradiation, and were further cultured for 24 h after the final irradiation. After fixation with cold methanol, intracellular CPs and nuclei were detected by fluorescence labeling with FTSC and Hoechst 33342, respectively. (a) Intracellular CPs were quantified by measuring F.I. after fluorescence labeling with FTSC, calculated as the value of F.I. divided by the F.I. of nuclei stained with Hoechst 33342. Intracellular CPs levels are expressed as fold changes normalized with intracellular CPs of untreated sham-UVA irradiated NHDFs. UVA irradiation is abbreviated as UVA R. Data are expressed as means $\pm$ standard deviations ($n=5$). □ ; sham-UVA R, ■ ; UVA R. (b) Representative images of intracellular CPs. UVA irradiation is abbreviated as UVA R. Scale bars: 100 μm . Significance; *** $p < 0.001$.

Fig. 3 Restorative effects of OC and RA on MMP-1 altered by repetitive UVA irradiation. NHDFs were irradiated with UVA at a dose of 10 J/cm^2 once a day for 3 successive days. Cells were cultured in the presence or absence of OC (250 μg/mL) or RA (62.5 μg/mL) after each UVA irradiation, and were further cultured for 24 h (mRNA) or 48 h (protein) after the final irradiation. (a) mRNA expression levels of MMP-1 induced by UVA irradiation in NHDFs. mRNA expression levels are expressed as means $\pm$ standard deviations ($n=3$). UVA irradiation is abbreviated as UVA R. □ ; sham-UVA R, ■ ; UVA R. (b) Representative images of WBs of MMP-1 proteins secreted from NHDFs irradiated with UVA. (c) MMP-1 levels were digitalized from images of WBs with Image J, and are expressed as means $\pm$ standard deviations ($n=3$). □ ; sham-UVA R, ■ ; UVA R. Significance; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

levels of type I collagen in sham-UVA irradiated NHDFs (Fig. 4). Although treatment with OC or RA increased the mRNA expression level of COL1A1 (Fig. 4a), the level of type I collagen protein in NHDFs treated with OC or RA did not show any increase (Fig. 4b). However, treatment with OC or RA significantly restored protein levels of type I collagen which were decreased by repetitive UVA irradiation (Fig. 4b).

3.4 The effects of OC and RA on the formation of collagen fibers by NHDFs exposed repetitively to UVA

NHDFs produced type I collagen fibers during 14 d of culture, but repetitive UVA irradiation decreased the formation of type I collagen fibers. The restoration effects of OC and RA on collagen fiber formation by NHDFs following repetitive UVA irradiation were then examined. Treatment with OC or RA significantly improved the decreased formation of type I collagen fibers induced by repetitive UVA irradiation (Fig. 5).

Fig. 4 Restorative effects of OC and RA on type I collagen altered by repetitive UVA irradiation. NHDFs were irradiated with UVA at a dose of 10 J/cm² once a day for 3 successive days. Cells were cultured in the presence or absence of OC (250 µg/mL) or RA (62.5 µg/mL) after each UVA irradiation, and were further cultured for 24 h (mRNA) or 48 h (protein) after the final irradiation. (a) mRNA expression levels of type I collagen induced by UVA irradiation are expressed as means ± standard deviations (n = 4). UVA irradiation is abbreviated as UVA R. □ ; sham-UVA R, ■ ; UVA R. (b) Protein levels of type I collagen were quantified with ELISA, and are expressed as means ± standard deviations (n = 4). □ ; sham-UVA R, ■ ; UVA R. Significance; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Fig. 5 Effects of OC and RA on type I collagen fiber formation by repetitive UVA irradiation. NHDFs were irradiated with UVA at a dose of 10 J/cm² once a day for 3 successive days. Cells were cultured in the presence or absence of OC (250 µg/mL) or RA (62.5 µg/mL) after each UVA irradiation and then were further cultured for 2 w. After fixation with 4% formaldehyde, collagen fibers were immunostained with anti-type I collagen antibody and anti-rabbit IgG (H + L) antibody-conjugated Alexa Fluor 594. (a) Representative images of collagen fibers, (b) Areas of type I collagen fibers were digitalized with corneocytometry (n = 3). Scale bar: 100 µm. Areas of type I collagen fibers are expressed as means ± standard deviations. UVA irradiation is abbreviated as UVA R. □ ; sham-UVA R, ■ ; UVA R. Significance; *** $p < 0.001$.

4 Discussion

Exposure to sunlight, which includes UVB and UVA, initiates and promotes photoaged skin characterized by deep wrinkles and sags. It has been demonstrated that a fragile dermal structure, including the loss of collagen fibers due to the altered function of fibroblasts, is responsible for the formation of wrinkles and sags. The loss of collagen fibers in the upper dermis is caused by an imbalance between the synthesis and degradation of collagen fibers triggered by ROS. On the other hand, among the different types of UV,

it is recognized that UVA is the major generator of ROS in fibroblasts and in the dermis, because UVB hardly penetrates into the dermis¹. The mechanism by which ROS causes the imbalance between the synthesis and degradation of collagen fibers has been well demonstrated as follows; ROS increases the production of Cyr61/CCN-1 (cysteine-rich angiogenic inducer 61), which is an internal suppressor of collagen synthesis¹³, and MMP-1, which is a collagenase that degrades type I fibrillar collagens¹⁴, through the activation of epidermal growth factor-receptor

signaling¹⁵⁾. Furthermore, $^1\text{O}_2$ generated through the photosensitization reaction by UVA stimulates the biosynthesis of MMP-1 in fibroblasts through the signaling of IL-1 and IL-6¹⁶⁾. Summarizing those facts, the elevation of intracellular ROS, for example, by stimulation with UVA, results in the depletion of collagen fibers due to an imbalance of its synthesis and degradation in the dermis. Thus, in order to estimate the potential of an effective anti-photoaging material, restoration of the imbalance between collagen synthesis and MMP-1 through the reduction of intracellular ROS is usually examined. Thus, in this study, the potential of OC, which contains RA, as an anti-photoaging material was investigated focusing on the restoration of the imbalance between the synthesis and degradation of collagen based on the reduction of intracellular ROS and the formation of collagen fibers impaired by repetitive UVA irradiation. Regarding UVB, it is presumed that substances secreted from UVB-exposed keratinocytes are causative factors in the imbalance between the synthesis and degradation of collagen fibers, since fibroblasts are not directly exposed to UVB. The effects of OC or RA on the altered function of fibroblasts initiated by substances secreted from UVB-exposed keratinocytes will be reported elsewhere after continuing the study.

Although it has been reported that OC contains antioxidant components in addition to RA, such as linalool, eugenol, methyl chavicol, methyl cinnamate, ferulate methyl eugenol, triterpenoids and steroidal glycoside¹⁷⁾, OC and RA did not show higher scavenging abilities against ROS from the chemical aspect (Table 1). However, from the biological aspect, both OC and RA showed significant reductions of levels of ROS and intracellular CPs in fibroblasts that were elevated by a single exposure to UVA (Fig. 1). Furthermore, OC and RA showed significant reductions of CPs in repetitively UV irradiated fibroblasts, which is a model of chronic sun-exposure (Fig. 2). The sum of these results indicates that OC and RA reduce intracellular oxidative stress biologically regardless of their relatively poor scavenging abilities from the chemical aspect.

Regarding the imbalance between collagen synthesis and MMP-1 production in fibroblasts repetitively irradiated with UVA, OC and RA significantly restored both of them at the protein level (Fig. 3, 4). However, OC and RA gave conflicting results between mRNA and protein levels involved in the synthesis of type I collagen. Despite upregulating the mRNA expression level of COL1A1 in sham-UVA irradiated fibroblasts, OC and RA did not show any changes in the level of type I collagen protein (Fig. 4). Since type I collagen is composed of COL1A1 and COL1A2 chains¹⁸⁾, it might be presumed that OC and RA did not have an effect on the mRNA expression level of COL1A2. However, both OC and RA restored the imbalance between the synthesis and degradation of type I collagen induced by repetitive UV irradiation, because protein levels are a more critical

issue than mRNA levels. Furthermore, OC and RA significantly improved collagen fiber formation that was impaired by repetitive UVA irradiation (Fig. 5). Collagen fibers are constructed by the cross-linking of triple-helical collagen peptides, COL1A1 and COL1A2, by enzymatic reaction with lysyl oxidase (LOX) after secretion¹⁹⁾. In a preliminary examination, we found that fibroblasts exposed to oxidative stress had a downregulation of LOX mRNA expression (data not shown). The restoration effects of OC and RA on collagen fiber formation might be involved in improving the change of LOX protein. We will continue further studies to demonstrate the restoration effects of OC and RA on collagen fiber formation in more detail and will report those elsewhere.

5 Conclusion

This study confirmed that treatment with OC or RA reduced levels of intracellular ROS and intracellular CPs in UVA irradiated fibroblasts, which resulted in the improvement of collagen fibers formed by UVA irradiated fibroblasts. It was considered that the reduction of intracellular oxidative stress by OC and RA is responsible for those improvements. In addition, based on the effective concentrations of OC and RA, the results suggest that RA is one of the active components in OC.

Taken together, these results demonstrate that OC, which contains RA, improves or prevents photoaged skin by restoring collagen fiber formation that is impaired by repetitive UVA irradiation.

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