Available online at www.sciencedirect.com
ScienceDirect
Journal of Nutritional Biochemistry 20 (2009) 389–398
Journal of Nutritional Biochemistry

Peroxidized cholesterol-induced matrix metalloproteinase-9 activation and its suppression by dietary β-carotene in photoaging of hairless mouse skin

Yuko Minami[a], Kyuichi Kawabata[a], Yoshiaki Kubo[b], Seiji Arase[b], Katsuya Hirasaka[c], Takeshi Nikawa[c], Noriko Bando[a], Yoshichika Kawai[a], Junji Terao[a,*]

[a]*Department of Food Science, Graduate School of Nutrition and Bioscience, the University of Tokushima, Tokushima 770-8503, Japan*
[b]*Department of Dermatology, Graduate School of Medical Science, the University of Tokushima, Tokushima 770-8503, Japan*
[c]*Department of Nutritional Physiology, Graduate School of Nutrition and Bioscience, The University of Tokushima, Tokushima 770-8503, Japan*

Received 24 March 2008; received in revised form 16 April 2008; accepted 22 April 2008

Abstract

The activation of matrix metalloproteinase (MMP)-9 leading to the formation of wrinkle and sagging of skin is an essential step in the skin photoaging on exposure to ultraviolet A (UVA). This study attempted to elucidate the role of peroxidized cholesterol including cholesterol hydroperoxides (Chol-OOHs), primary products of lipid peroxidation in biomembranes, in MMP-9 activation and the effect of dietary β-carotene in MMP-9 activation. Hairless mice were subjected to periodic UVA irradiation for 8 weeks. The amount of peroxidized cholesterol detected as total hydroxycholesterol in the skin was increased significantly by the exposure. The activity and protein level of MMP-9 were elevated with wrinkling and sagging formation. MMP-9 activity was also enhanced by the intracutaneous injection of Chol-OOHs into the mouse skin. Adding β-carotene to the diet of the mice during the period of irradiation suppressed the activity and expression of MMP-9 as well as the wrinkling and sagging formation. The amount of cholesterol 5α-hydroperoxide, a singlet molecular oxygen oxygenation-specific peroxidized cholesterol, was significantly lowered by the addition of β-carotene to the diet. These results strongly suggest that Chol-OOHs formed on exposure to UVA contribute to the expression of MMP-9, resulting in photoaging. Dietary β-carotene prevents the expression of MMP-9, at least partly, by inhibiting photodynamic action involved in the formation of Chol-OOHs.

Keywords: Skin; Photoaging; UVA; β-Carotene; Cholesterol hydroperoxides; Matrix metalloproteinase-9

1. Introduction

Sunlight, particularly long-wavelength ultraviolet (UV) light (UVA), is suggested to reach the dermis and cause oxidative damage by generating reactive oxygen species (ROS) via photodynamic actions [1]. UVA-induced oxidative damage is characteristic of skin photoaging, which is distinguished from physiological aging in terms of morphological changes [2,3]. For example, the degradation of collagen and elastin leading to an enlargement of the dermis with wrinkles and sagging is a hallmark of photoaging [4]. Members of the matrix metalloproteinase (MMP) family, in particular, MMP-1, MMP-2 and MMP-9, are implicated in skin photoaging [5]. Experiments using cultured cell lines demonstrated that these MMPs are activated in response to attack by ROS [6,7]. In addition, the expression of MMPs was found to proceed through a mitogen-activated protein kinase (MAPK) pathway [8], which is known to be activated by ROS and ROS-induced oxidative stress [9]. However, no exact mechanism for the activation of MMPs by attack of ROS was clarified yet.

ROS generated in the skin are capable of reacting with a wide variety of biological components, in which unsaturated lipids constituting cellular and subcellular biomembranes seem to be responsible for disruptions of cellular function.

Abbreviations: UVA, ultraviolet A; MMP, matrix metalloproteinase; Chol 7α-OOH, cholesterol 7α-hydroperoxide; Chol 7β-OOH, cholesterol 7β-hydroperoxide; Chol 5α-OOH, cholesterol 5α-hydroperoxide; ROS, reactive oxygen species; LA-OOH, linoleic acid hydroperoxide; 4-HNE, 4-hydroxynonenal; St-OOH, sitosterol hydroperoxide; Cu-OOH, cumene hydroperoxide; GC–MS/SIM, gas chromatography–mass spectrometry/selected ion monitoring; HPLC, high-performance liquid chromatography; MAPK, mitogen-activated protein kinase; H&E, hematoxylin and eosin; O_2 ($^1\Delta_g$), singlet molecular oxygen; HRP, horseradish peroxidase.

* Corresponding author. Tel.: +81 88 633 7087; fax: +81 88 633 7089.
E-mail address: terao@nutr.med.tokushima-u.ac.jp (J. Terao).

0955-2863/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.jnutbio.2008.04.010

Cholesterol is a lipid constituent of biomembranes, and its primary peroxidation products generated by reactions with ROS, cholesterol hydroperoxides (Chol-OOHs), are suggested to be a biomarker of physiological aging in the skin [10]. Yamazaki et al. [11] and our group [12] also found that Chol-OOH levels in the skin of hairless mice were elevated by UV irradiation. In cellular membranes, cholesterol is an intrinsic part of rafts where the cellular signaling pathway starts to convey signals toward the nucleus [13]. It was also reported that cholesterol plays an essential role in the expression of MMPs through the modulation of MAPK signaling [14]. Therefore, peroxidized cholesterol in the biomembranes may play a role in the activation of MMPs in skin photoaging.

β-Carotene and other carotenoids are dietary antioxidants by acting as singlet molecular oxygen [O2 ($^1\Delta_g$)] quenchers and unique free radical scavengers [15,16]. Our ex vivo study clarified that dietary β-carotene is capable of quenching O2 ($^1\Delta_g$) generated in UVA irradiation of mouse skin homogenate [17]. On the other hand, several studies have demonstrated that dietary β-carotene protects human skin from the UV-light-induced erythema [18–20]. However, little is known on the protective effect of dietary β-carotene in UVA-induced skin photoaging.

The aim of this study is to evaluate the relationship between the formation of cholesterol peroxidation products and the activation of MMP-9 in UVA-dependent photoaging. In addition, the photoprotective effect of dietary β-carotene was investigated to know the physiological significance of β-carotene accumulated in the skin. Hairless mice subjected to periodic UVA irradiation were examined for morphological changes on the back with the measurement of MMP-9 activity and Chol-OOHs. The effect of Chol-OOHs on the activation of MMP-9 was compared with those of cholesterol and other lipid peroxidation-related products by their intracutaneous injections into the mouse skin. An experiment using mice fed a β-carotene-containing diet was carried out to estimate the efficacy of dietary β-carotene in MMP-9 activation. The results indicate that Chol-OOHs generated by photodynamic action in the skin are involved in the promotion of photoaging through the induction of MMP-9 and that dietary β-carotene suppresses MMP-9 activation effectively.

2. Materials and methods

2.1. Materials

β-Carotene, β-apo-8′-carotenal, α-tocopherol and δ-tocopherol were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). β-Sitosterol hydroperoxide (St-OOH) and Chol-OOHs were prepared from hematoporphyrin-sensitized photooxidation of β-sitosterol (Tama Biochemical, Tokyo, Japan) and cholesterol (Kanto Chemical Co., Tokyo, Japan), respectively, according to the method described in a previous paper [12]. Linoleic acid hydroperoxide (LA-OOH) was also prepared through methylene-blue-sensitized photooxidation of linoleic acid (Sigma-Aldrich Co.), as described elsewhere [21]. Cumene hydroperoxide (Cu-OOH) was purchased from Sigma-Aldrich Co. 4-Hydroxynonenal (4-HNE) was obtained from Cayman Chemical (Michigan, USA). Hydrogen peroxide was the product of Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The anti-MMP-9 antibodies were purchased from Sigma-Aldrich Co. The horseradish peroxidase (HRP)-conjugated anti-rabbit antibody was obtained from Cell Signaling Technology Inc. (Beverly, MA). β-Actin was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). HRP-conjugated anti-mouse and anti-goat antibodies were from Dako Co. (Glostrup, Denmark). All other reagents and solvents were of guaranteed reagent grade from Kanto Chemical Co.

2.2. Animal study design

Five-week-old male Hos;HR-1 hairless mice were purchased from SLC Japan (Hamamatsu, Japan) and maintained under standard experimental conditions (25°C, 60% humidity; light and dark cycle every 12 h) in accordance with the Guidelines for Animal Experimentation of Tokushima University. In Experiment I, mice were randomly divided into two groups (n=8 in each group) and fed the control diet [corn starch 40, casein 20, sucrose 20, corn oil 5, lard 5, cellulose 4.75, mineral mixture (oriental mixture) 3.5, vitamin mixture (oriental mixture) 1, D,L-methionine 0.3, sodium cholate 0.25, choline chloride 0.2 g/100 g diet]. All mice were given free access to food and water for 11 weeks. The first 3 weeks were set aside for acclimatization. In the subsequent 8 weeks, the mice were treated with UVA irradiation (+UVA group) or else not irradiated (−UVA group). In Experiment II, mice were again randomly divided into two groups (n=5 in each group) and fed the control diet (control group) or a β-carotene-supplemented diet (β-carotene 50, α-tocopherol 5 mg/100 g control diet; BC group). Our preliminary experiment showed that the level of α-tocopherol in the skin was decreased by a single dose of β-carotene. Thus, a mixture of β-carotene and α-tocopherol was added as a dietary supplement to adjust the amount of α-tocopherol in the skin. Both groups were allowed free access to food and water for 11 weeks. The first 3 weeks were set aside for acclimatization. The subsequent 8 weeks were for UVA irradiation. For the measurement of the amounts of β-carotene and α-tocopherol accumulated in the mouse skin without UVA irradiation, each group (n=5 in each group) was also fed the control diet or the β-carotene-supplemented diet and allowed free access to food and water for 11 weeks without the treatment of UVA irradiation. In all experiments, mice were eventually anesthetized with sodium pentobarbital (50 mg/kg) and sacrificed by exsanguination from the heart.

2.3. UVA irradiation

The protocol for the irradiation was approved by the ethics committee for animal experiments of Tokushima

University. Mice were exposed to irradiation from a UVA projection lamp (FL15BL-B, National Inc., Osaka, Japan; maximum wavelength at 352 nm, with range of 315–380 nm) five times a week for 8 weeks. The UVA dose was set to 3.9 J/cm^2 for the initial 4 weeks and then increased weekly by 5 J/cm^2, 10 J/cm^2 up to 15 J/cm^2 and finally held at 15 J/cm^2 for 2 weeks based on Kim et al. [22] (Fig. 1). The distance between the lamp and animal was approximately 12 cm, and the light's intensity was adjusted to 1.3 mW/cm^2 by measuring with a UV radiometer (UM-10, Minolta Co., Ltd., Japan). Mice moved freely in the cages during the period of exposure. A piece of skin, 3.0×4.0 cm, was obtained from the middorsal region so as to avoid contamination by hypodermic fats and stored at −80°C prior to the analysis.

2.4. Histological and morphological analyses of mouse skin

For hematoxylin and eosin (H&E) staining of the skin tissue, a part of the middorsal skin sample (1×0.3 cm) was fixed in a 10% formalin neutral buffer solution and embedded in paraffin blocks. Serial sections (4 μm) were mounted onto MAS-coated slides and stained with H&E after dewaxing. For estimating UVA-induced morphological changes of the skin, a wrinkle score was measured according to the method of Bissett et al. [4]. A pinch test was also adopted as an index of skin sagging using the method of Tsukahara et al. [23]. Briefly, mice were anesthetized with sodium pentobarbital (50 mg/kg) and the dorsal skin was lifted up with the fingers as much as possible and the pinch was subsequently released. The time until the skin recovered to the original state was measured.

2.5. Quantification of peroxidized cholesterol in mouse skin

The skin sample was added to nine volumes of phosphate-buffered saline and homogenized with a Polytron homogenizer (Kinematica AG, Littau/Luzern, Switzerland). The skin lipids were extracted from the homogenates by the method of Bligh and Dyer [24] after the addition of a known amount of St-OOH as an internal standard. Peroxidized cholesterol was reduced to hydroxycholesterol and isolated by DPPP-TLC blotting as previously described [12]. Briefly, the lipid extract was applied to a TLC plate (silica gel 60 F_{254}, 0.25 mm thickness; Merck) and developed with 93:7 (v/v) hexane–isopropanol as mobile phase. The TLC plate was then dipped in 0.01% (w/v) DPPP-containing blotting solvent (isopropanol–0.2% aqueous $CaCl_2$–methanol, 40:20:7, v/v/v) for 30 s, after which it was placed on a glass fiber filter. Then, the plate was covered with a PVDF membrane, a PTFE membrane and, finally, a glass fiber filter, in layers. This TLC-blotting sandwich was pressed evenly for 90 s at 180°C. After blotting, the spot whose R_F value corresponded to standard Chol-OOHs was cut out and immersed in chloroform–methanol (1:1, v/v). The solvent was then removed with nitrogen gas. Then, gas chromatography–mass spectrometry/selected ion monitoring (GC–MS/SIM) was applied to the hydroxycholesterol fractions after their conversion to trimethylsilyloxyl derivatives.

2.6. Measurement of the amounts of β-carotene and α-tocopherol in mouse skin

The skin sample was added to nine volumes of phosphate-buffered saline and homogenized with a Polytron homogenizer to determine the level of β-carotene in mouse skin. Skin homogenates were mixed with 5 μl of 10 mM butyl hydroxytoluene in hexane and 100 pmol apo-8′-carotenal as an internal standard. For the determination of α-tocopherol, the homogenates were mixed with 10 μl of 1 μM δ-tocopherol as an internal standard. Then, levels of β-carotene and α-tocopherol were determined by high-performance liquid chromatography (HPLC) [17].

2.7. Treatment of mouse skin with Cu-OOH, Chol-OOHs and related compounds

Male Hos;HR-1 hairless mice (8 weeks old) were intracutaneously injected with the following test compounds into the back under anesthesia with sodium pentobarbital (50 mg/kg): 1 or 20 nmol of Cu-OOH, 1 nmol of Chol-OOH or cholesterol, 1 nmol of LA-OOH, 1 nmol of 4-HNE and 1 nmol of hydrogen peroxide. The injection points were four

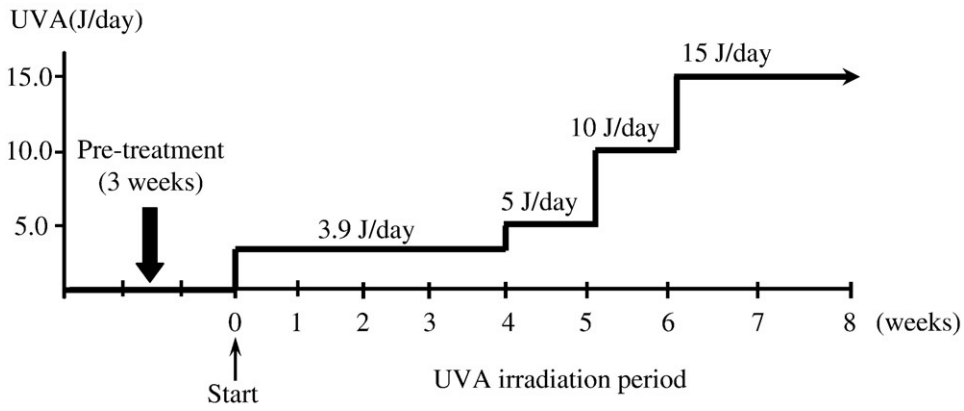

Fig. 1. Schedule of periodic UVA irradiation to the hairless mice.

or five sites on the back in each mouse. Each part of the dorsal skin was collected at 24 h after the treatment and stored at −80°C prior to the measurement of MMP-9 activity.

2.8. Gelatin zymography for the measurement of MMP-9 activity

The skin sample was added to four volumes of phosphate-buffered saline containing 4% (v/v) protease inhibitor cocktail and 10% (v/v) phosphatase inhibitor cocktail (Roche, Indiana, USA) and homogenized with a Polytron homogenizer. Levels of MMP-9 activity in the skin homogenate were determined as proMMP by gelatin zymography as described previously [25]. Briefly, the homogenates were centrifuged at 7000×g for 10 min at 4°C and soluble fractions were collected. Then, protein concentrations were determined by the bicinchoninic acid assay using a BCA™ protein assay kit (PIERCE, USA). The skin samples were mixed 1:1 with sample buffer (4% SDS, 125 mM Tris–HCl, 20% glycerol and 0.1% bromophenol blue), and equal amounts of protein (25 μg) were analyzed for activity to degrade gelatin by SDS-PAGE under nonreduced conditions using 10% polyacrylamide containing 0.6 mg/ml gelatin. After the electrophoresis, the gel was washed with washing buffer [50 mM Tris, 5 mM calcium chloride, 1 μM zinc chloride, 0.02% (w/v) sodium azide, 2.5% (v/v) Triton X-100 and 6 mg/ml heparin] with gentle shaking for 1 h at room temperature. Next, the gel was incubated in incubation buffer [50 mM Tris, 5 mM calcium chloride, 1 μM zinc chloride and 0.02% (w/v) sodium azide] at 37°C for 24 h. Proteolytic activity was visualized by staining with 0.25% (w/v) Coomassie brilliant blue R250 in 50% methanol, 10% acetic acid and distilled water for 30 min at room temperature and destaining with 1% acetic acid, 30% methanol and distilled water. The intensity of bands was quantified with NIH image software.

2.9. Western blotting for the measurement of MMP-9 expression

The skin sample was added to four volumes of phosphate-buffered saline containing 4% (v/v) protease inhibitor cocktail (Roche) and 10% phosphatase inhibitor cocktail (Roche) and then homogenized with a Polytron homogenizer. The homogenates were centrifuged (7000×g, 10 min, 4°C) and the protein content of the supernatant was quantified using a BCA® protein assay kit. Samples were mixed 1:1 with sample buffer (125 mM Tris, pH 6.8, 4% SDS, 20% glycerol and 10% 2-mercaptoethanol), and equal amounts of sample proteins (50–60 μg) were separated on 10% polyacrylamide gels under reduced conditions and transferred electrophoretically onto Immobilon-P membranes (Millipore, Billerica, MA). Following blocking using SuperBlock® Blocking Buffer (PIERCE) for 20 min at room temperature, the membrane was incubated with rabbit anti-MMP-9 antibody (1:1000) or with goat anti-β-actin antibody (1:1000) overnight at 4°C. Then, the membrane was incubated with HRP-conjugated anti-rabbit IgG (1:2000) or anti-goat IgG (1:2000). The bands were developed using ECL Western blotting detection reagent, and their intensity was analyzed using NIH image. Relative levels of MMP-9 were corrected with β-actin as the internal standard.

3. Results

3.1. Morphological and histological changes of the mouse skin

Fig. 2 shows the back of mice of the −UVA and +UVA groups. Large, deep wrinkles were observed after irradiation for 8 weeks (Fig. 2A and B). H&E staining of skin sections indicated an increase in the thickness of the dermis and epidermis and clear augmentation of cysts in the dermis in the +UVA group. Wrinkling was markedly increased after 5 weeks of irradiation (Fig. 2C), while sagging was enhanced from the initial stages of irradiation (Fig. 2D). These results confirmed that this periodic UVA irradiation promoted skin photoaging, as these morphological and histological changes are characteristic of such [26–28].

3.2. Effect of periodic UVA irradiation on enzyme activity and protein expression of MMP-9

Gelatin zymography was used to evaluate the effect of UVA irradiation for 8 weeks on the activity of MMP-9 in the skin, measured as bands of proMMP-9 (Fig. 3A). The irradiation enhanced the activity of MMP-9. Fig. 3B shows the effect on the expression of MMP-9 proteins. Western blotting indicated that UVA irradiation promoted the expression of MMP-9 proteins.

3.3. Effect of periodic UVA irradiation on the accumulation of peroxidized cholesterol in the skin

Free cholesterol accounts for 7.5% of human skin lipids [29]. In the hairless mice, cholesterol was found to compose 9% of skin lipids, as the skin contained total lipids at 88 mg/g skin and total skin contained cholesterol at 8 mg/g skin. We measured the level of peroxidized cholesterol accumulated in the skin using a combination of TLC blot and GC–MS/SIM [12]. Total hydroxycholesterol [Chol-(O)OH] derived from Chol-OOH and its reduced derivative, Chol-OH, was detected as peroxidized cholesterol in the SIM chromatogram as shown in Fig. 4. This chromatogram indicates that cholesterol 5α-hydroperoxide (Chol 5α-OOH) and cholesterol 7α/β-hydroperoxide (Chol 7α/β-OOH) were formed in the hairless mouse skin. Table 1 shows the amount of peroxidized cholesterol in the skin of hairless mice with and without UVA irradiation. The levels of Chol 5α-(O)OH and Chol 7α/β-(O)OH were significantly higher in the group exposed to periodic UVA irradiation (+UVA group).

3.4. Enhancement of MMP-9 activity by intracutaneous injection of Cu-OOH, cholesterol/Chol-OOH and related compounds

To clarify the effect of the products of lipid peroxidation accumulating in the skin on the MMP activity, we used

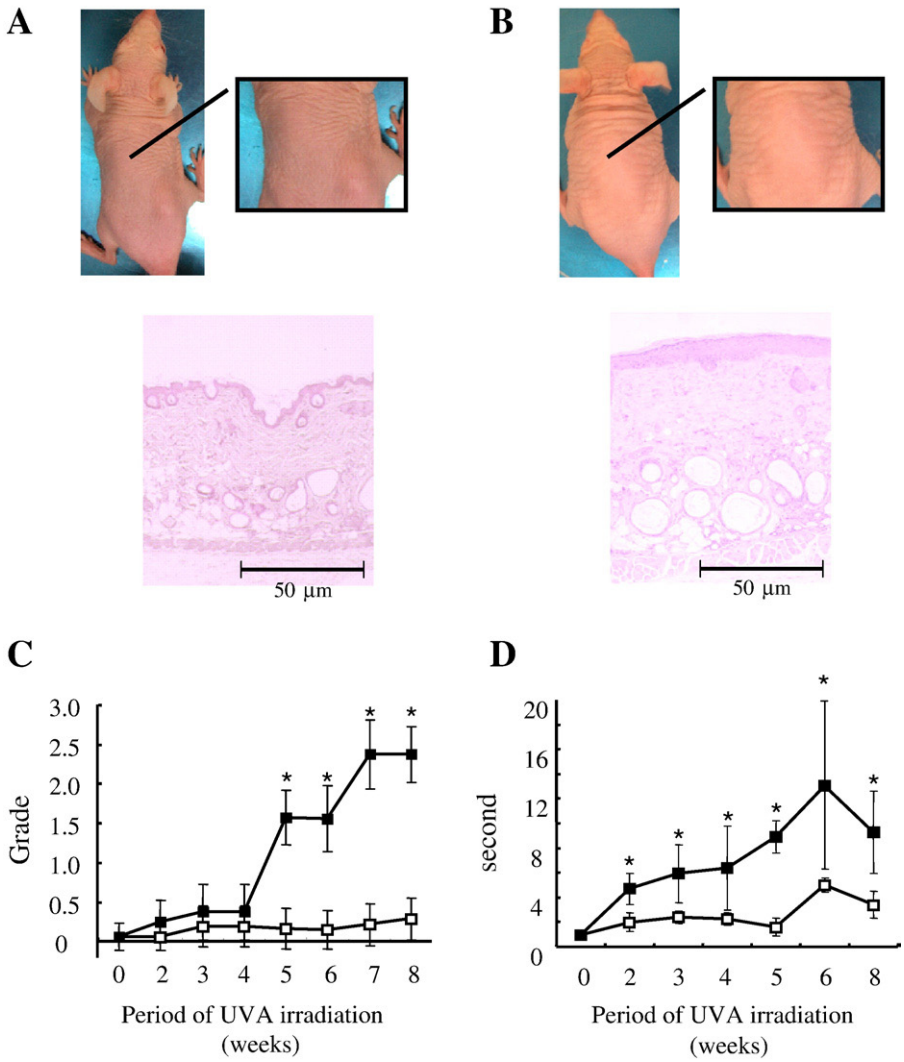

Fig. 2. The morphological and histological changes of mouse skin caused by UVA irradiation for 8 weeks. The +UVA group was exposed to UVA for 8 weeks (total, 303 J/cm^2). The −UVA group was not treated with UVA during this period. Pictures of the back and tissue sections of mice were taken (A, −UVA; B, +UVA). The histological changes to the skin were analyzed by H&E staining (scale bar=50 μm). (C) Wrinkle score. (D) Pinch testing. Open symbols, −UVA group; filled symbols, +UVA group. *Significantly different from the corresponding −UVA group (P<.05).

Cu-OOH as representative of such products and injected it intracutaneously into the back of hairless mice under anesthesia. Cu-OOH at both 1 and 20 nmol activated gelatinolytic activity of MMP-9 significantly at 24 h after the treatment as compared with the vehicle control (Fig. 5A). It is therefore evident that Cu-OOH can enhance the activity of MMP-9 in the skin. Chol-OOH at 1 nmol also activated MMP-9 twofold compared with vehicle control (Fig. 5B). Although slight activation of MMP-9 was observed with the injection of cholesterol itself, the enhancement by Chol-OOH was significantly greater than that by cholesterol (Fig. 5B). On the other hand, LA-OOH, 4-HNE, or hydrogen peroxide at 1 nmol did not affect the activity of MMP-9 (Fig. 5C). It is therefore likely that the enhancement of MMP-9 activity is more selective to peroxidized cholesterol than peroxidized fatty acids and their decomposition products when injected into the skin.

3.5. Effect of supplementing the diet with β-carotene on skin photoaging in hairless mice

Table 2 shows the levels of β-carotene and α-tocopherol in the skin of mice with and without periodic UVA irradiation for 8 weeks. Without irradiation, it was confirmed that the α-tocopherol level did not differ between the control group and the BC group. Interestingly, the β-carotene level was lowered after UVA irradiation in the BC group, indicating that β-carotene was consumed on exposure of UVA. Fig. 6A shows that the wrinkling and sagging were effectively suppressed with the addition of β-carotene to the diet. In addition, no change was observed in the H&E-

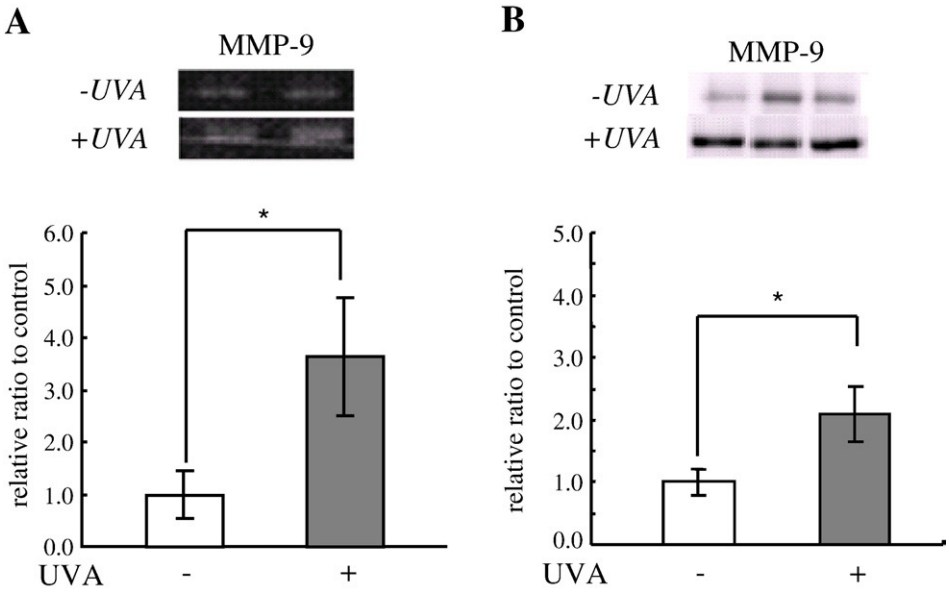

Fig. 3. MMP-9 activity and its expression in mouse skin with or without UVA irradiation. (A) Zymography for MMP-9 activity. (B) Western blotting for MMP-9 protein expression. The band intensity was expressed as relative to that of the −UVA group. Data are shown as the means±S.D. (n=4). Open bars, −UVA group; filled bars, +UVA group. *Significantly different from the corresponding −UVA group (P<.05).

stained skin tissue of the BC group after exposure (Fig. 6B). In contrast, an increase of epidermal thickness and the infiltration of inflammatory cells were found in the control group with periodic UVA irradiation. Fig. 6C shows the levels of peroxidized cholesterol accumulated in the skin in the BC group and control group after 8 weeks' irradiation. The level of Chol 5α-OOH in the skin of the BC group was significantly lower than that in the skin of the control group. Thus, it is likely that β-carotene in the skin inhibited UVA-induced accumulation of cholesterol peroxidation products. The activity and protein expression of MMP-9 in the UVA-irradiated skin were also reduced by supplementing the diet with β-carotene as shown in Fig. 6D. Therefore, it was found that dietary β-carotene was an effective agent for suppressing the promotion of photoaging by accumulating in the skin and inhibiting the expression of MMP-9.

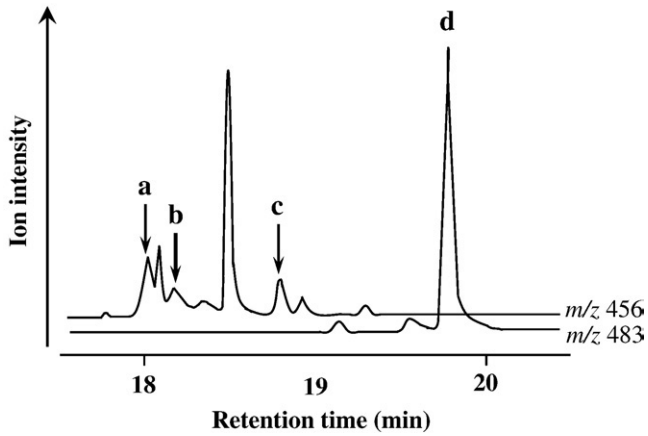

Fig. 4. GC–MS/SIM of peroxidized cholesterol obtained from skin in the +UVA group. Total lipids were extracted from the skin tissue and subjected to GC–MS/SIM after DPPP-TLC blotting. The SIM chromatogram was obtained by using specific fragment ions m/z 456 and m/z 483 from peroxidized cholesterol and the internal standard [St-(O)OH], respectively. Peaks identified are as follows: a, Chol 7α-(O)OH; b, Chol 5α-(O)OH; c, Chol 7β-(O)OH; d, St-(O)OH.

4. Discussion

It is known that the degradation of collagen by MMPs, mainly MMP-1, -2 and -9, is responsible for the formation of wrinkles and sagging of UVA-induced skin photoaging. The induction of MMP-9 expression by UVA irradiation is known to be an essential step in skin photoaging [30]. However, it is still unclear whether or not ROS generated by the irradiation and products of ROS-induced peroxidation participate in the induction process. Thus, we focused on MMP-9 activation to know whether its expression is induced by lipid peroxidation products derived from the reaction of ROS with skin lipids under UVA irradiation.

Table 1
The amount of peroxidized cholesterol in mouse skin with or without UVA irradiation

	Chol 7α-(O)OH	Chol 7β-(O)OH	Chol 5α-(O)OH
−UVA group (μmol/mol cholesterol)	0.45±0.11	0.63±0.06	0.32±0.02
+UVA group (μmol/mol cholesterol)	0.71±0.22*	0.99±0.26*	0.40±0.04*

Total lipids were extracted from skin tissue and subjected to DPPP-TLC blotting and GC–MS/SIM with the specific fragment ion m/z 456 [16]. Data are shown as the means±S.D. (n=5).

* Significantly different from the corresponding −UVA group (P<.05).

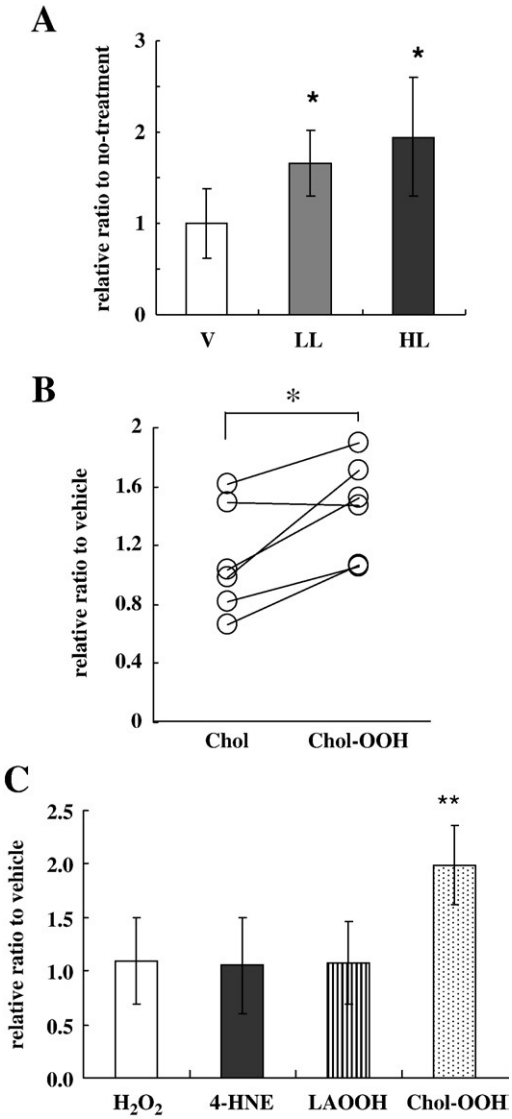

Fig. 5. The effects of intracutaneous injection of Cu-OOH (A), cholesterol/Chol-OOH (B) and related compounds (C) on MMP-9 activity. MMP-9 activity was analyzed by gelatin zymography using each part of the skin. Samples were recovered after treatment for 24 h. The protein in samples was extracted as described in Section 2. (A) Cu-OOH was injected into the back. V, injection of vehicle; LL, injection of 1 nmol Cu-OOH; HL, injection of 20 nmol Cu-OOH. The intensity of bands is shown relative to that for no-treatment. Data are expressed as the means±S.D. (n=3). ∗Significantly different from the corresponding nonirradiated group (P<.05). (B) Cholesterol (Chol) or Chol-OOH (1 nmol) was injected into the back. Chol-OOH consisted of Chol 5α-OOH (45.6%), Chol 7α-OOH (12.4%) and Chol 7β-OOH (45.6%). Data are expressed relative to the vehicle-treated control (means±S.D.; n=6). *Significantly different from cholesterol (P<.05). (C) Chol-OOH, LA-OOH, 4-HNE or hydrogen peroxide at 1 nmol was injected into the back. Open bar, H_2O_2; filled bar, 4-HNE; striped bar, LA-OOH; dotted bar, Chol-OOH. Data are expressed relative to the vehicle-treated control (means±S.D.; n=6). **Significantly different from the corresponding vehicle-treated control samples (P<.01).

In addition to the formation of deep, large wrinkles and sagging of the skin, histological analysis demonstrated events characteristic of photoaging, including the enlargement of the epidermis and infiltration of inflammatory cells in the dermis, in the skin of UVA-irradiated mice (Fig. 2). It was therefore ascertained that the UVA irradiation actually promoted skin photoaging as in previous reports [31,32]. MMPs are responsible for the degradation of collagen and elastin in dermis [33]. Therefore, their induction and/or activation are undoubtedly a major factor accelerating wrinkling and sagging of the skin. MMP-9, which acts as a gelatinase, especially decomposes Type IV collagen, elastin, Type I collagen and Type III collagen, constituting the basal membrane and dermis [34–36]. The degradation of collagen in the dermis and basal membrane by MMP-9 brings about the formation of deep, large wrinkles through disruption of the skin's structure [33]. It was reported that the expression of MMP-9 was induced by exposure to ROS including hydrogen peroxide and UV light in keratinocytes [6] and fibroblasts [7]. Thus, the induction of MMP-9 expression through ROS generated by UVA irradiation seems to be involved in the onset and progression of photoaging in the skin.

Currently, we found that UVA irradiation enhanced the activity of MMP-9. Levels of Chol-OOHs were increased in the mouse skin after UVA irradiation (Table 1). In particular, the finding that Chol 5α-OOH, a specific product for O_2 ($^1\Delta_g$) oxygenation [37], increased remarkably indicates that O_2 ($^1\Delta_g$) was generated in the skin by photodynamic action. In addition, Chol 7-α/β-OOH detected in the skin is known to be formed by the isomerization of Chol 5α-OOH as well as the reaction of free radicals with cholesterol [11]. We recently demonstrated that O_2 ($^1\Delta_g$) oxygenation occurred in skin lipids of hairless mice under ordinary feeding conditions and were accelerated by the exposure to UVA irradiation [38]. Wlaschek et al. [39] reported that the expression of MMP-1, an interstitial collagenase, increased on exposure to O_2 ($^1\Delta_g$) in human keratinocytes. Polte and Tyrrell [40] suggested that lipid peroxidation products formed by UVA irradiation induced the expression of MMP-1 in human fibroblasts. These reports prompted us to investigate whether or not O_2 ($^1\Delta_g$)-dependent lipid peroxidation products induce MMP-9 activity in vivo. Here, we injected a mixture

Table 2
The amounts of β-carotene and α-tocopherol in mouse skin with or without UVA irradiation

	Control		BC	
	−UVA	+UVA	−UVA	+UVA
β-Carotene (pmol/mg protein)	n.d.	n.d.	17.3±9.6	5.1±1.2*
α-Tocopherol (pmol/mg protein)	279±40	217±36	232±86	251±47

Mice were fed the control diet (control) or a β-carotene- and α-tocopherol-supplemented diet (BC) for 11 weeks. Each group was separated into a UVA-irradiated subgroup (+UVA) and a nonirradiated subgroup (−UVA). The irradiated subgroup was exposed to UVA for 8 weeks. The amounts of β-carotene and α-tocopherol in mouse skin were quantified by HPLC. Data are shown as the means±S.D. (n=5). n.d., not detected.

* Significantly different from the corresponding −UVA subgroup (P<.05).

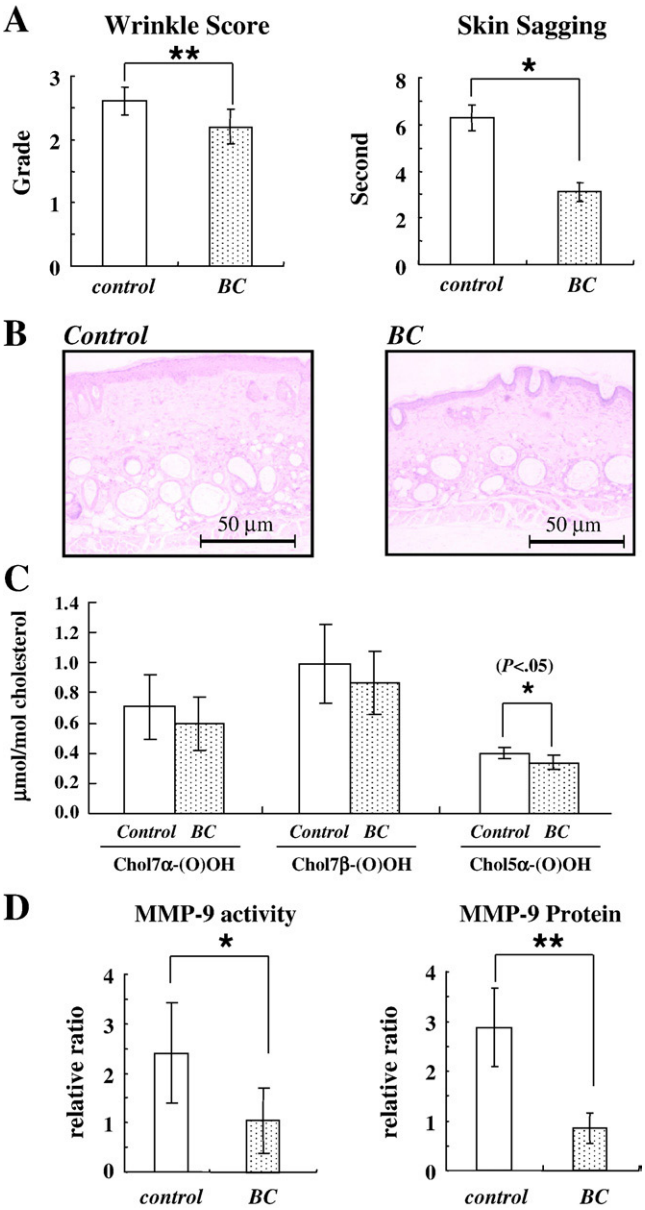

Fig. 6. Inhibition of skin photoaging in mice by the supplementation of β-carotene. (A) A wrinkle score and pinch test were measured for wrinkle formation and skin sagging, respectively. Open bars, control group with UVA irradiation; dotted bars, BC group with UVA irradiation. (B) H&E staining of skin section (scale bar=50 μm). (C) The amount of peroxidized cholesterol in mouse skin. Open bars, control group with UVA irradiation; dotted bars, BC group with UVA irradiation. Data are shown as the means± S.D. (n=5). Significantly different from the corresponding control with UVA irradiation (*P<.05). (D) The activity of MMP-9 and the expression of MMP-9 in mouse skin measured by gelatin zymography and Western blotting, respectively. The intensity of bands is shown relative to that for the control group without UVA irradiation. Open bars, control group with UVA irradiation; dotted bars, BC group with UVA irradiation. Data are shown as the means±S.D. (n=5). Significantly different from the corresponding control with UVA irradiation (*P<.05, **P<.01).

of Chol-OOH isomers prepared from the products of photosensitized oxidation of cholesterol intracutaneously into the skin of mice to test their ability to enhance MMP-9 activity. The result clearly shows that an isomeric Chol-OOH mixture is able to enhance MMP-9 activity, when injected into the skin (Fig. 5B and C). The mixture consisted of Chol 5α-OOH (45.6%), Chol 7α-OOH (12.4%) and Chol 7β-OOH (42.0%). It is not known whether or not O_2 ($^1\Delta_g$) oxygenation-derived Chol 5α-OOH is more active than the other isomers in the enhancement of MMP-9 activity. Nevertheless, the same result was obtained in the experiment using Cu-OOH as a model of lipid hydroperoxide (Fig. 5A). Thus, these results imply that organic hydroperoxide is generally capable of inducing MMP-9. In fact, neither 4-HNE, a reactive secondary product of lipid peroxidation, nor hydrogen peroxide, an inorganic hydroperoxide, exhibited any effect on MMP-9 activity in the skin of live rodents (Fig. 5C), although both of these products were reported to induce MMP expression in cultured cell lines [7,41,42]. However, LA-OOH, a primary product of the peroxidation of linoleic acid, did not enhance MMP-9 activity under these experimental conditions (Fig. 5C). Higher reactivity of LA-OOH to detoxification by cellular glutathione peroxidase Type I [43] might be related to its lack of efficacy on MMP-9 activity. Esterified hydroperoxides such as phospholipid hydroperoxides are likely to be also involved in the enhancement agents, as they are resistant to cellular glutathione peroxidase Type I as similarly to Chol-OOHs. Alternatively, Chol-OOHs may exert MMP-9 activation specifically by affecting rafts where cellular signaling pathway for MMP activation starts. Further study is needed to clarify the precise mechanism through which the MMP-9 is activated by Chol-OOH and other lipid peroxidation products accumulated in skin tissue.

An experiment in which the diet for mice was supplemented with β-carotene was undertaken to estimate its protective effect on UVA-induced skin photoaging. β-Carotene can act as a potent quencher of O_2 ($^1\Delta_g$) in phospholipid bilayers of biomembranes [44,45], and our previous ex vivo study clarified that dietary β-carotene inhibited O_2 ($^1\Delta_g$) oxygenation of unsaturated lipids in the homogenate of mouse skin exposed to UVA [17]. This in vivo study clarified that dietary β-carotene is capable of suppressing MMP-9 expression as well as inhibiting morphological changes in the skin.

Furthermore, adding β-carotene to the diet evidently suppressed the accumulation of O_2 ($^1\Delta_g$) oxygenation-specific cholesterol peroxidation product, Chol 5α-OOH (Fig. 6C), indicating that dietary β-carotene actually quenches O_2 ($^1\Delta_g$) to prevent the formation of peroxidized cholesterol in the UVA-irradiated skin. α-Tocopherol is also capable of quenching O_2 ($^1\Delta_g$) in biomembranes [46,47]. However, the fact that the content of α-tocopherol in the skin of the BC group was not different from that of the control group (Table 2) suggests that α-tocopherol scarcely participates in the inhibition of MMP-9 activation by β-carotene supplementation. Although the O_2 ($^1\Delta_g$) quenching activity of β-carotene is mostly attributable to its physical quenching without a chemical reaction, a chemical reaction with β-carotene also happens during the physical quenching

[48]. A decrease of β-carotene content in the skin by the exposure of UVA (Table 2) may have partly originated from the chemical reaction of β-carotene with O_2 ($^1\Delta_g$). Thus, it is indicated that the intake of dietary β-carotene is able to suppress photodynamic action involving O_2 ($^1\Delta_g$) oxygenation in UVA-exposed skin and is, therefore, promising in the prevention of skin photoaging. It is also plausible that O_2 ($^1\Delta_g$) is included in ROS responsible for the enhancement of MMP-9 in UVA-exposed skin.

In conclusion, Chol-OOHs generated by photodynamic action possess a potential to induce the expression of MMP-9 leading to skin photoaging under UVA irradiation. It is clarified that dietary β-carotene is helpful in preventing UVA-induced skin photoaging by suppressing MMP-9 activation, at least partly, through the inhibition of photodynamic action occurring in the skin.

Acknowledgments

This study was partly supported by the Ministry of Education, Culture, Sports, Science and Technology program of the 21st Century Center of Excellence "Human nutritional science on stress control." We thank Ms. K. Yokoyama for technical assistance.

References

[1] Bruls WA, Van Weedlden H, Van der Leun JC. Transmission of UV-irradiation through human epidermal layers as a factor influencing the minimal erythema dose. Photochem Photobiol 1984;39:63–7.
[2] El-Domyati M, Attia S, Saleh F, Brown D, Birk DE, Gasparro F, et al. Intrinsic aging vs. photoaging: a comparative histopathological, immunohistochemical, and ultrastructural study of skin. Exp Dermatol 2002;11:398–405.
[3] Yasui H, Sakurai H. Age-dependent generation of reactive oxygen species in the skin of live hairless rats exposed to UVA light. Exp Dermatol 2002;12:655–61.
[4] Bissett DL, Hannon DP, Orr TV. An animal model of solar-aged skin: histological, physical, and visible changes in UV-irradiated hairless mouse skin. Photochem Photobiol 1987;46:367–78.
[5] Rittie L, Fisher GJ. UV-light-induced signal cascades and skin aging. Ageing Res Rev 2002;1:705–20.
[6] Kawaguchi Y, Tanaka H, Okada T, Konishi H, Takahashi M, Ito M, et al. The effects of ultraviolet A and reactive oxygen species on the mRNA expression of 72-kDa type IV collagenase and its tissue inhibitor in cultured human dermal fibroblasts. Arch Dermatol Res 1996;288:39–44.
[7] Brenneisen P, Briviba K, Wlaschek M, Wenk J, Scharffetter-Kochanek K. Hydrogen peroxide (H_2O_2) increases the steady-state mRNA levels of collagenase/MMP-1 in human dermal fibroblasts. Free Radic Biol Med 1997;22:515–24.
[8] Zeigler ME, Chi Y, Schmidt T, Varani J. Role of ERK and JNK pathways in regulating cell motility and matrix metalloproteinase 9 production in growth factor-stimulated human epidermal keratinocytes. J Cell Physiol 1999;180:271–84.
[9] Nelson KK, Melendez JA. Mitochondrial redox control of matrix metalloproteinases. Free Radic Biol Med 2004;37:768–84.
[10] Tahara S, Matsuo M, Kaneko T. Age-related changes in oxidative damage to lipids and DNA in rat skin. Mech Ageing Dev 2001;122:415–26.
[11] Yamazaki S, Ozawa N, Hiratsuka A, Watanabe T. Quantitative determination of cholesterol 5alpha-, 7alpha-, and 7beta-hydroperoxides in rat skin. Free Radic Biol Med 1999;27:110–8.
[12] Minami Y, Yokoi S, Setoyama M, Bando N, Takeda S, Kawai Y, et al. Combination of TLC blotting and gas chromatography–mass spectrometry for analysis of peroxidized cholesterol. Lipids 2007;42:1055–63.
[13] Helms JB, Zurzolo C. Lipids as targeting signals: lipid rafts and intracellular trafficking. Traffic 2004;5:247–54.
[14] Kim S, Kim Y, Lee Y, Cho KH, Kim KH, Chung JH. Cholesterol inhibits MMP-9 expression in human epidermal keratinocytes and HaCaT cells. FEBS Lett 2007;581:3869–74.
[15] Paiva SA, Russel RM. β-Carotene and other carotenoids as antioxidants. J Am Coll Nutr 1999;18:426–33.
[16] Krinsky NI. Carotenoids as antioxidants. Nutrition 2001;17:815–7.
[17] Bando N, Hayashi H, Wakamatsu S, Inakuma T, Miyoshi M, Nagao A, et al. Participation of singlet oxygen in ultraviolet-A-induced lipid peroxidation in mouse skin and its inhibition by dietary beta-carotene: an ex vivo study. Free Radic Biol Med 2004;37:1854–63.
[18] Stahl W, Heinrich U, Jungmann H, Sies H, Tronnier H. Carotenoids and carotenoids plus vitamin E protect against ultraviolet light-induced erythema in humans. Am J Clin Nutr 2000;71:795–8.
[19] Heinrich U, Gartner C, Wiebusch M, Eichler O, Sies H, Tronnier H, et al. Supplementation with β-carotene or a similar amount of mixed carotenoids protects humans from UV-induced erythema. J Nutr 2003;133:98–101.
[20] Lee J, Jiang S, Levine N, Watson RR. Carotenoid supplementation reduces erythema in human skin simulated solar radiation exposure. Proc Soc Exp Biol Med 2000;223:170–4.
[21] Terao J, Asano I, Matsushita S. Preparation of hydroperoxy and hydroxy derivatives of rat liver phosphatidylcholine and phosphatidylethanolamine. Lipids 1985;20:312–7.
[22] Kim HH, Lee MJ, Lee SR, Kim KH, Cho KH, Eun HC, et al. Augmentation of UV-induced skin wrinkling by infrared irradiation in hairless mice. Mech Ageing Dev 2005;126:1170–7.
[23] Tsukahara K, Nakagawa H, Moriwaki S, Kakuo S, Ohuchi A, Takema Y, et al. Ovariectomy is sufficient to accelerate spontaneous skin ageing and to stimulate ultraviolet irradiation-induced photoageing of murine skin. Br J Dermatol 2005;151:984–94.
[24] Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. Can J Biochem Physiol 1959;37:911–7.
[25] Kawabata K, Murakami A, Ohigashi H. Auraptene decreases the activity of matrix metalloproteinases in dextran sulfate sodium-induced ulcerative colitis in ICR mice. Biosci Biotechnol Biochem 2006;70:3062–5.
[26] Kambayashi H, Yamashita M, Odake Y, Takada K, Funasaka Y, Ichihashi M. Epidermal changes caused by chronic low-dose UV irradiation induce wrinkle formation in hairless mouse. J Dermatol Sci 2001;27:S19–S25.
[27] Bhawan J, Andersen W, Lee J, Labadie R, Solares G. Photoaging versus intrinsic aging: a morphologic assessment of facial skin. J Cutan Pathol 1995;22:154–9.
[28] Kligman LH. The ultraviolet-irradiated hairless mouse: a model for photoaging. J Am Acad Dermatol 1989;21:623–31.
[29] Reinertson RP, Wheatley VR. Studies on the chemical composition of human epidermal lipids. J Invest Dermatol 1959;32:49–59.
[30] Krutmann J. Ultraviolet A radiation-induced biological effects in human skin: relevance for photoaging and photodermatosis. J Dermatol Sci 2000;23:S22–6.
[31] Moloney SJ, Edmonds SH, Giddens LD, Learn DB. The hairless mouse model of photoaging: evaluation of the relationship between dermal elastin, collagen, skin thickness and wrinkles. Photochem Photobiol 1992;56:505–11.
[32] Ogawa F, Sato S. Roles of oxidative stress in photoaging and the pathogenesis of systemic sclerosis. Nihon Rinsho Meneki Gakkai Kaishi 2006;29:349–58.
[33] Inomata S, Matsunaga Y, Amano S, Takada K, Kobayashi K, Tsunenaga M, et al. Possible involvement of gelatinases in basement membrane damage and wrinkle formation in chronically ultraviolet B-exposed hairless mouse. J Invest Dermatol 2003;120:128–34.

[34] Murphy G, Cockett MI, Ward RV, Docherty AJ. Matrix metalloproteinase degradation of elastin, type IV collagen and proteoglycan. A quantitative comparison of the activities of 95 kDa and 72 kDa gelatinases, stromelysins-1 and -2 and punctuated metalloproteinase (PUMP). Biochem J 1991;277:277–9.
[35] Okada Y, Gonoji Y, Naka K, Tomita K, Nakanishi I, Iwata K, et al. Matrix metalloproteinase 9 (92-kDa gelatinase/type IV collagenase) from HT 1080 human fibrosarcoma cells. Purification and activation of the precursor and enzymic properties. J Biol Chem 1992;267:21712–9.
[36] Senior RM, Griffin GL, Fliszar CJ, Shapiro SD, Goldberg GI, Welgus HG. Human 92- and 72-kilodalton type IV collagenases are elastases. J Biol Chem 1991;266:7870–5.
[37] Yamazaki S, Ozawa N, Hiratsuka A, Watabe T. Photogeneration of 3beta-hydroxy-5alpha-cholest-6-ene-5-hydroperoxide in rat skin: evidence for occurrence of singlet oxygen in vivo. Free Radic Biol Med 1999;27:301–8.
[38] Minami Y, Yokoyama K, Bando N, Kawai Y, Terao J. Occurrence of singlet oxygen oxygenation of oleic acid and linoleic acid in the skin of live mice. Free Radic Res 2008;42:197–204.
[39] Wlaschek M, Briviba K, Stricklin GP, Sies H, Scharffetter-Kochanek K. Singlet oxygen may mediate the ultraviolet A-induced synthesis of interstitial collagenase. J Invest Dermatol 1995;104:194–8.
[40] Polte T, Tyrrell RM. Involvement of lipid peroxidation and organic peroxides in UVA-induced matrix metalloproteinase-1 expression. Free Radic Biol Med 2004;36:1566–74.
[41] Akiba S, Kumazawa S, Yamaguchi H, Hontani N, Matsumoto T, Ikeda T, et al. Acceleration of matrix metalloproteinase-1 production and activation of platelet-derived growth factor receptor beta in human coronary smooth muscle cells by oxidized LDL and 4-hydroxynonenal. Biochim Biophys Acta 2006;1763:797–804.
[42] Morquette B, Shi Q, Lavigne P, Ranger P, Fernandes JC, Benderdour M. Production of lipid peroxidation products in osteoarthritic tissues: new evidence linking 4-hydroxynonenal to cartilage degradation. Arthritis Rheum 2006;54:271–81.
[43] Thomas JP, Maiorino M, Ursini F, Girotti AW. Protective action of phospholipid hydroperoxide glutathione peroxidase against membrane-damaging lipid peroxidation. In situ reduction of phospholipid and cholesterol hydroperoxides. J Biol Chem 1990;265:454–61.
[44] Stratton SP, Liebler DC. Determination of singlet oxygen-specific versus radical-mediated lipid peroxidation in photosensitized oxidation of lipid bilayers: effect of β-carotene and α-tocopherol. Biochemistry 1997;36:12911–20.
[45] Fukuzawa K, Inokami Y, Tokumura A, Terao J, Suzuki A. Rate constants for quenching singlet oxygen and activities for inhibiting lipid peroxidation of carotenoids and alpha-tocopherol in liposomes. Lipids 1998;33:791–6.
[46] Di Mascio P, Devasagayam TP, Kaiser S, Sies H. Carotenoids, tocopherols and thiols as biological singlet molecular oxygen quenchers. Biochem Soc Trans 1990;18:1054–6.
[47] Fukuzawa K, Matsumura K, Tokumura A, Suzuki A, Terao J. Kinetics and dynamics of singlet oxygen scavenging by alpha-tocopherol in phospholipid model membranes. Free Radic Biol Med 1997;22:923–30.
[48] Foote CS, Denny RW. Chemistry of singlet oxygen. VII. Quenching by β-carotene. J Am Chem Soc 1968;90:6233–5.